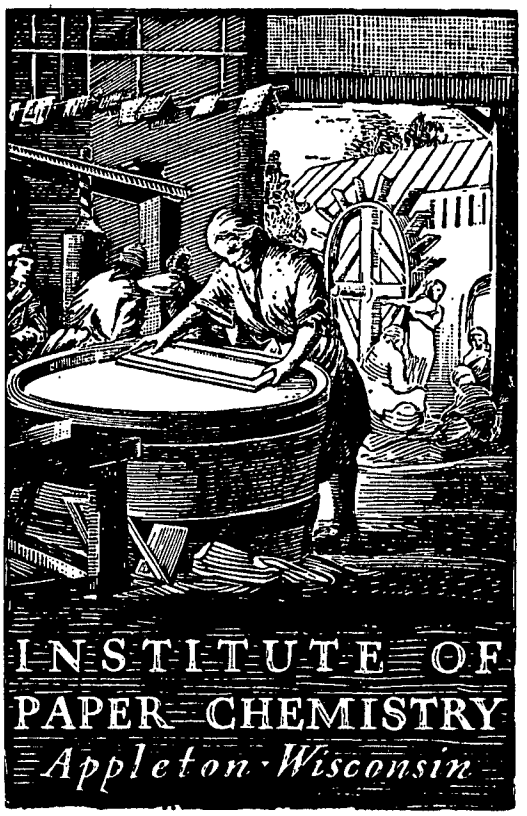

THE MASS PRODUCTION OF CONIFER TREE HYBRIDS PHASE I. DEVELOPMENT OF CELL SUSPENSION–EMBRYOID–PLANTLET TECHNIQUE

Project 3223

Report Four
A Progress Report
to
MEMBERS OF THE INSTITUTE OF PAPER CHEMISTRY

July 1, 1976

THE INSTITUTE OF PAPER CHEMISTRY

Appleton, Wisconsin

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THE MASS PRODUCTION OF CONIFER TREE HYBRIDS PHASE I. DEVELOPMENT OF CELL SUSPENSION — EMBRYOID — PLANTLET TECHNIQUE

SUMMARY

The level of activity for the tissue culture and biochemical phases of the program remained very high during the past six months. Investigations on morphology and the ultrastructure of differentiating tissue and developing embyroids were postponed in an effort to concentrate remaining funds and other expenditures on the "embryoid production" phase of the research program. Ultrastructure investigations are expected to increase in early fall as more advanced types of structures are produced.

Douglas-fir shoots were produced from several types of plant material using tissue culture techniques. When seed embryos were removed and placed on a simplified agar growth medium, single vigorous shoots appeared on the tip of the swollen seed leaves (cotyledons). Increasing cytokinin levels resulted in the production of smaller multiple shoots from seed leaves. When the seed leaves from Douglas-fir seeds were removed and placed on a growth medium containing auxin and cytokinin, callus was produced. After subculturing the callus several times (monthly), shoots began to develop. Eleven of 35 subcultured sources of seed leaf callus produced shoots. Single shoots were also produced from subcultured needle callus and callus on an isolated stem section (explant) of seed leaf callus origin.

Difficulties encountered in establishing long-term loblolly pine callus cultures resulted in the establishment of a series of experiments designed to optimize the growth medium for loblolly pine. Factors being considered and varied included inorganic salts, vitamins, amino acids, and growth hormones as well as

light and temperature. An improved growth medium has been developed and is being tested with further modifications being considered.

Embryoid initiation studies were also undertaken using Douglas-fir stem callus that had been grown in the light for 6-7 years. Such callus is generally believed to be too old to produce shoots unless rejuvenated in some manner. The use of varying hormone levels, light conditions (day length and intensity), nitrogen sources and cold treatments were employed to stimulate embryoid production in callus. The most dividing E-cells were obtained when the above callus was transferred from 5000 lux light to 2000 lux fluorescent light (16 hour day at 24°C and 8 hour dark at 18°C). Varying the levels of the hormone 2,4-D influenced the growth and physiological condition, with levels below 5 mg/liter producing healthy green tissue. Varying combinations of BAP and NOAA also influenced growth and general callus condition, with a 1:1 BAP/NOAA ratio and levels of 1 mg/liter each being one combination that was satisfactory. To date the cold treatments tried resulted in no significant differences between treatments.

Enzyme investigations during the past several months have centered on peroxidases, catalase, indoleacetic acid (IAA) oxidases, phenol oxidases, superoxide dismutases, and polyamine oxidases. Relationships between enzymes and the degree of cellular organization coupled with recent positive "feedback" responses emphasized the importance of enzymes in growth and differentiation of callus and suspension cultures. A substantial number of enzyme distribution patterns have been developed for several enzymes and for stem callus, cotyledon callus and organized tissue. Variables investigated included age, growth medium, and genetic source. Peroxidase-catalase patterns, for example, appear to be a function of age, growth medium, clonal differences, and numbers of times subcultured with time

of assay after subculture particularly noteworthy for catalase activity. Similar variables were examined in the cases of phenol oxidase and superoxide dismutase.

Other results of interest concerning enzyme activity were the low apparent IAA oxidase activity in Douglas-fir stem callus and the apparent inability of Douglas-fir stem callus to oxidize polyamines. This latter observation is particularly interesting because one of the products of polyamine oxidase action should be hydrogen peroxide, the oxidizing agent in peroxidase activity and an effective agent in feedback experiments. Furthermore, the biosynthesis of polyamines could proceed from arginine via ornithine, both of which are also compounds of current interest.

Thin-layer chromatography continued to be used as a tool in comparative analyses of low molecular weight compounds. Of major interest was the discovery that what was considered to be lysine and arginine in organized tissue was really ornithine and arginine. Because, theoretically, ornithine can be formed from arginine by a one-step enzymatic reaction, its presence rather than lysine sheds new light on the whole metabolic picture.

Nitrogen sources are being investigated in connection with both callus and cell suspension growth. Results with stem callus indicate that, when ammonium nitrate is used, the stem callus is using only the ammonium portion of the nitrogen supply. Early results with nitrogen sources demonstrated that the ammonium form of nitrogen was required to grow cells in the light. Recent results suggest that when ammonium is used, growth appears to be satisfactory but embryoid development may be inhibited. This information is being rechecked and several alternative sources of nitrogen have been investigated, the best of which seems to be urea.

A series of studies were initiated to investigate the effect of auxin (2,4-D) on growth of Douglas-fir cell suspensions and embyroid formation. The results indicate the cells in suspension are very sensitive to auxin levels with levels from 0.1 to 1.0 mg/liter producing increasing numbers of green cells, E-cells and embryoids.

The possibility that ammonium ions may inhibit cell differentiation in Douglas-fir cell suspensions was investigated by the use of other inorganic and organic forms of nitrogen. Although differences were not large, evidence was found that ammonium ions appeared to be inhibiting differentiation and, as a result, a urea type of nitrogen was selected for use in Douglas-fir cell suspensions being used to produce embryoids.

A number of internal and external factors influencing growth and embyroid development in cell suspensions have been studied over the past several months. Effects of initial pH, removal of inhibitors, types of growth vessels, sieving of cell clusters, types of agitation, nutrient renewal frequency, and light and temperature were considered. General recommendations are given on conditions and techniques for use with several sources of callus and types of growth media. In related cell suspension studies, suspensions prepared from callus derived from new (subcultured only 9-11 times) sources of Douglas-fir and loblolly pine produced larger and greater numbers of embryoids than cell suspensions derived from callus that had been subcultured for a number of years.

Feedback research has also received a modest amount of attention during the past six months. Ten milliliter suspension cultures were used to test the effect of chemical additives on growth and organization. Thiourea and hydrogen peroxide were used to test the hypothesis that excessively high catalase activity

may be inhibiting organization. Similarly arginine and urea were tested as alternate sources of nitrogen. While neither hydrogen peroxide nor thiourea consistently stimulated E-cell or embryoid formation better than the standard growth media employed, both additives produced E-cells at shorter times than standard media and stimulated greater cell differentiation in the presence of urea than in the presence of nitrate and ammonium nitrogen. Both arginine and urea were found to serve as sources of nitrogen. Whether they will be better than presently employed sources remains to be seen.

INTRODUCTION

The history of Project 3223, and the increasing interest by the paper industry in the use of tissue culture techniques as a method of vegetatively propagating trees, makes an interesting story. Institute Project 3223, Mass Production of Conifer Hybrids, was initiated on July 1, 1974, after a number of years' experience with tissue culture propagation of aspen and approximately three years' experience in a less intensive way with several species of conifers. The interest, controversy and discussions associated with the establishment of Project 3223 has resulted in increased interest and, more importantly, increased tissue culture research at several other institutions. We feel this increased effort, using a variety of approaches, is an extremely healthy situation and is one that will facilitate maximum progress. The development of tissue culture techniques as a reliable complementary method to conventional tree improvement methods is highly desirable.

The research team involved in Project 3223 (Drs. M. Johnson, Parham and Winton) is following a combination of empirical and biochemical methods. We believe this approach will result in maximum progress toward the development of a reliable mass production technique. The "callus" approach as a primary goal continues to be deemphasized, with major emphasis being directed toward working out a "single cell-embryoid-plantlet" procedure. Recent promising enzyme and biochemical results are beginning to fit together with earlier observations and are providing important feedback information regarding the biochemical pathways of the system under investigation. The report that follows describes progress during the past six months toward the overall goal of the mass production of conifer hybrids.

GROWTH AND ORGANIZATION OF CALLUS

SHOOTS FROM DOUGLAS-FIR CULTURES*

Abstract

Douglas-fir [Pseudotsuga menziesii (Mirb.) Franco] embryos were excised from the same seed lot and placed on a simplified medium made without auxin but containing 0.05 mg/liter BAP (benzylaminopurine). After 3-4 weeks, usually only one vigorous shoot appeared from the tip of each of the swollen cotyledons. When 0.1 mg/liter BAP was added, small multiple shoots appeared all over the cotyledons. When embryos or cotyledons with shoots were transferred from the 0.05 BAP treatment to medium without hormones, the shoots elongated and produced normal appearing needles. However, the small multiple shoots from the 0.1 BAP treatment usually did not elongate normally.

On the other hand, when excised embryos were started on a medium without hormones, and the cotyledons were subsequently excised and placed on medium having both an auxin and a cytokinin, the cotyledons swelled and produced callus that was isolated and subcultured monthly. From 53 seeds we established 35 clonal cultures of subcultured cotyledon callus. Shoots were produced from 11 of the 35 clones, for a frequency of 31%, indicating a high genetic variability among seed genomes in their ability to produce shoots from callus.

Of the many shoots excised and placed in rooting medium, two rooted after treatment with 10 mg/liter IBA. In addition to embryo material, one shoot was

*This section was submitted to the Canadian Journal of Botany for publication by L. Winton and S. Verhagen. This paper is based on data reported in Project 3223 Progress Report Three, January 30, 1976, and will be presented at the Fourth North American Forest Biology Workshop, August 9-11, 1976, at Syracuse State University, New York.

produced from needle callus subcultured three times and one shoot was produced from callus on a stem explant prepared from a cotyledon callus shoot.

Introduction

At present, the aseptic culture of conifer trees permits the clonal propagation of seeds and seedlings of only a few species, but indicates the future potential for the mass production of genetically identical ramets from older seedlings and trees of most conifers (1-4). Methods of propagation from callus will also be necessary for the regeneration of plants from the products of somatic hybridization, involving either the fusion of protoplasts from species of the same genus or from transformed protoplasts which have received genetic information from species of a different genus or family (4-5).

So far, buds and leafy shoots have been produced from cultures of relatively few conifer tree species (Table I) and plantlets have been reported only from cotyledons of Pinus palustris (6-7), hypocotyls of Picea glauca (8), and from cotyledons and subcultured cotyledon callus of Pseudotsuga menziesii (9).

This paper reports methods of shoot production from cotyledons and subcultured cotyledon callus of Douglas-fir different from those cited above, including a measure of variability among seeds to produce shoots from cotyledon callus. We also report the production of leafy shoots from subcultured needle callus and stem explant callus.

Methods and Results

Seeds of Douglas-fir [Pseudotsuga menziesii (Mirb.) Franco], Lot 491-15-1, were received in February, 1974, from Dr. John Rediske, Weyerhaeuser Company, Centralia, Washington, and were surface sterilized either by a quick dip in 95% alcohol or for 15 minutes in half-strength bleach containing 2.5% sodium hypochlorite.

TABLE I

CONIFER TREE SPECIES PRODUCING BUDS, SHOOTS OR PLANTLETS

Species & Reference	Treated Material	Results[a]
Biota orientalis[b] (10)	Cotyledon	Shoots
Cryptomeria japonica (11-14)	Hypocotyl	Shoots
Picea abies (15,16)	Stem callus	Shoots
Picea glauca (8)	Hypocotyl	Plantlets
Pinus banksiana (16,17)	Hypocotyl callus	Shoots
Pinus cembra	Hypocotyl callus	Shoots
Pinus elliottii (7)	Cotyledon	Shoots
Pinus lambertiana (18)	Hypocotyl, hypocotyl callus	Shoots
Pinus palustris	Cotyledon	Plantlets
Pinus ponderosa (16)	Stem callus	Buds
Pinus rigida (7)	Cotyledon	Shoots
Pinus strobus (7)	Cotyledon, hypocotyl callus	Shoots
Pinus taeda (7)	Cotyledon	Shoots
Pinus virginiana (7)	Cotyledon	Shoots
Pseudotsuga menziesii (16)	Stem callus	Buds
(9)	Cotyledon	Shoots
(9)	Cotyledon callus	Plantlets
Sequoia sempervirens (19)	Stem callus	Buds

[a] Buds = nonfunctional structures a few mm long. Shoots = elongating structures with normal appearing needles. Plantlets = excised and rooted shoots.

[b] Genus *Biota* is now genus *Thuja*.

Seeds sterilized in bleach were then rinsed several times and stored overnight in sterile water at 3°C. Seed embryos were excised and placed at 5-7 per Petri dish on an agar medium suitable to the experiment. The dishes were sealed with Parafilm and the embryos were grown for one week in the dark at 27°C, then transferred to an incubator having 2000 lux of cool-white fluorescent light for 16 hours at 24°C, alternating with 8 hour darkness at 18°C.

Shoots Directly from Cotyledons

Seeds were sterilized with bleach, and embryos were excised and placed on medium E-1 or 19 (Table II) containing 0.05 or 0.1 mg/liter BAP (benzylaminopurine). After one week in the dark, embryos were transferred to the light incubator where they swelled to several times their original diameter and turned green, but did not elongate as did normal seedlings on the same medium without BAP. There was no significant difference between medium E-1 and 19, but a large difference was observed btween the two levels of BAP.

On medium with 0.05 mg/liter BAP, usually only one shoot appeared at the tip of most cotyledons after 3-4 weeks. Most cotyledons were excised and transferred to medium without hormones, where the shoots elongated and produced normal appearing needles. Figure 1 shows a whole embryo after nine weeks on medium 19 with 0.05 mg/liter BAP. This embryo was transferred intact to medium without hormones, to a light source in the laboratory having 4000 lux fluorescent and 400 lux tungsten light, on for 16 hours and dark for 8 hours, at 23-25°C. Figure 2 shows the embryo three months after transfer, when two shoots had elongated to 2-3 cm.

When excised embryos were placed on medium with 0.1 mg/liter BAP, 50-60% produced small multiple shoots all over their cotyledons after 3-5 weeks. About

TABLE II

NUTRIENT MEDIA USED TO CULTURE DOUGLAS-FIR

A. Salts common to all media[a]

Component	Mg/liter	Component	Mg/liter
NH_4NO_3	1,650	H_3BO_3	6.2
KNO_3	1,900	KI	0.83
$MgSO_4$	370	Na_2MoO_4	0.25
KH_2PO_4	170	$CuSO_4$	0.025
$CaCl_2$	440	$CoCl_2$	0.025
$MnSO_4$	16.9	Fe(EDTA)[b]	5.6
$ZnSO_4$	10.6		

B. Components in mg/liter added to common salts to give specific media

Component	Medium 10[c]	Medium 19	Medium E-1
Nicotinic acid	0.5	--	--
Thiamine	0.1	--	0.4
Pyridoxine	0.1	--	--
Inositol	100	--	100
Asparagine	100	--	--
BAP[d]	0.1	--	--
NOAA[d]	5	--	--
Sucrose	30,000	30,000	40,000
Agar	8,000	8,000	8,000

[a] Adapted from Brown & Lawrence (20). Water of hydration not given.

[b] 5 Ml of stock solution containing 5.57 g $FeSO_4$ and 7.45 g Na_2-EDTA per liter of H_2O.

[c] From Winton (21).

[d] BAP = N^6-benzylaminopurine, NOAA = naphthoxyacetic acid.

10% of the embryos were stunted and had very tiny multiple shoots only at the tips of their cotyledons. However, when whole embryos, excised cotyledons, or cotyledon tips were transferred to medium without hormones and placed in fluorescent-tungsten light, the small multiple shoots rarely elongated normally, if at all (Fig. 3).

In this study, many more shoots were produced with the higher level of BAP, but the only shoots to elongate normally were the fewer and more vigorous shoots initiated at the lower level of BAP.

Shoots from Subcultured Cotyledon Callus

Seeds were sterilized with a quick dip in 95% ethanol and the embryos were excised as soon as the alcohol evaporated. Embryos were placed on medium E-1 in the dark and, after one week, were transferred to the light incubator where the cotyledons began to elongate and turn green. After 3-4 weeks on medium E-1, the cotyledons were 1-2 cm long and were excised and placed on medium 10 (Table II) containing both an auxin and a cytokinin.

After a few weeks on medium 10, the cotyledons became covered with soft green callus, which was isolated from the original cotyledon tissue and sub-cultured monthly to fresh medium 10. Over a period of several passages the callus became darker green in color and firmer in texture, and all callus derived from one embryo was labelled as a separate clonal culture.

Over a period of 15 months in 1974-75, attempts were made to grow callus from 53 seed embryos. Eighteen callus clones were lost because of poor growth or contamination, and 35 clones were established as subcultured callus cultures. Of the 35 clones, 11 started to produce shoots after 1-6 subcultures, giving a frequency of 31% of the surviving clones that produced shoots (Table III).

TABLE III

SHOOTS FROM DOUGLAS-FIR SUBCULTURED COTYLEDON CALLUS

Number	Clone	Embryos Excised	Number of Subcultures[a]	First Shoots 1974	First Shoots 1975
1	9	4- 1-74	5	11-18	1-28
2	10	4- 1-74	4	11-19	
3	34	5-21-74	3	11-19	
4	42	7-25-74	2	12- 4	3- 3
5	46	8-26-74	2		2- 3[b]
6	49	8-26-74	6		6-13
7	51	10- 5-74	4		6- 4
8	52	10-14-74	1		1-13
9	58	4-23-75	3		9-26
10	62	4-23-75	2		8-25
11	67	7- 3-75	2		11-11

[a] Number of subcultures until shoots first appeared.
[b] First shoots to elongate more than 1 cm while attached to callus.

Generally, only one vigorous shoot appeared on a callus piece and on 1-2 callus pieces per dish of 5 (Fig. 4), but occasionally 2-3 shoots grew from one callus. Shoots were initiated over a period of several months from individual clones, but no attempt was made to accurately record the number of shoots produced. However, a conservative estimate would be 100-200 shoots from all 11 clones.

Many callus pieces were transferred to medium E-1 without hormones, and shoots elongated and produced normal appearing needles. Figure 5 shows one callus piece with several elongating shoots, after several months in fluorescent-tungsten light. Many elongating shoots 1-5 cm tall were excised from callus and placed in

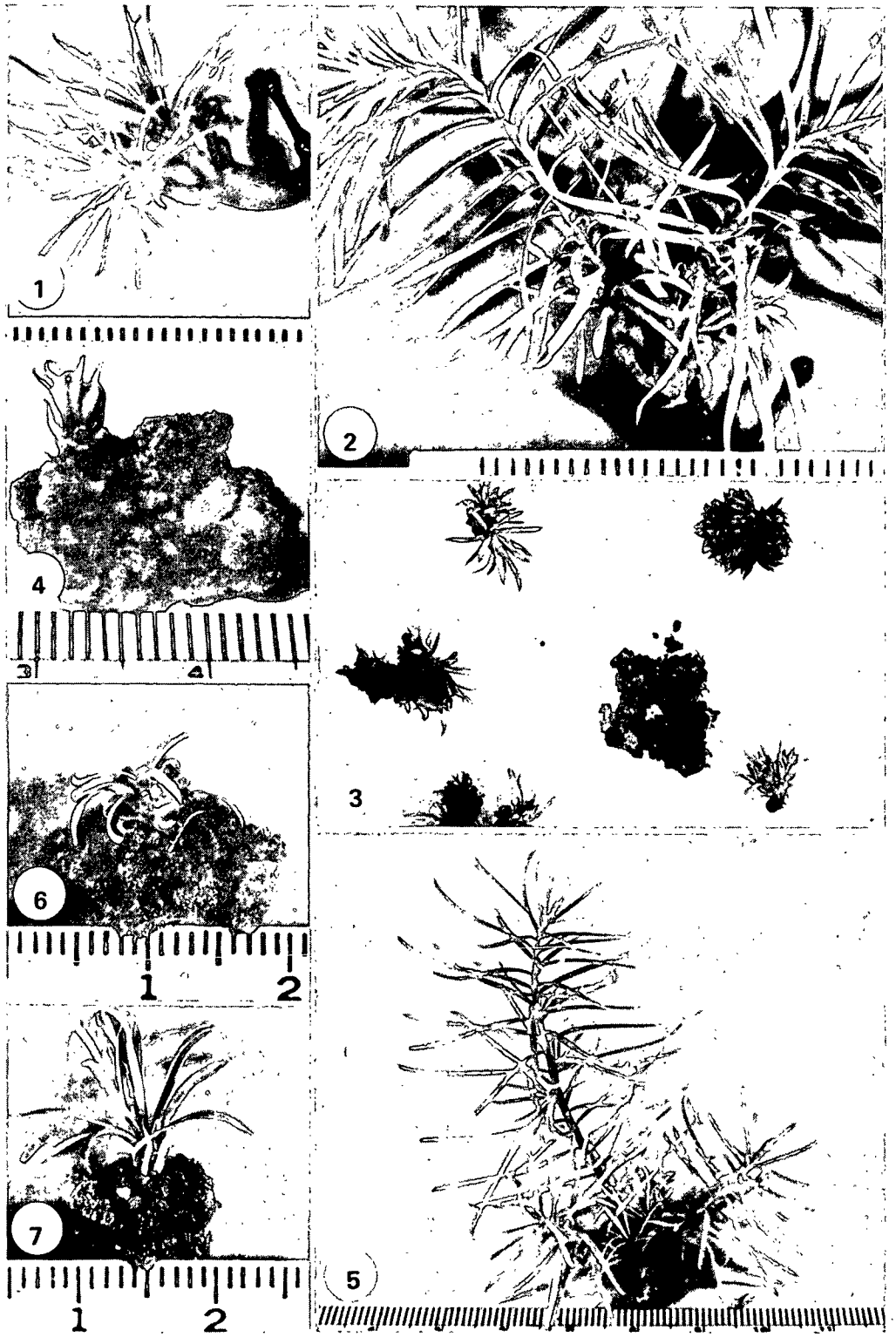


Figure 1. Swollen Douglas-fir Seed Embryo After Nine Weeks on Medium with 0.05 mg/liter BAP. Single Leafy Shoots Grew from the Tips of Most Cotyledons. Figure 2. The Same Embryo Shown in Fig. 1 was Transferred to Medium without Hormones, and is Shown Here After Three Months when Two Shoots Elongated to 2-3 cm. Figure 3. Small Multiple Shoots were Initiated over the Surface of Excised Cotyledons on Medium 19 with 0.1 mg/liter BAP, and are Shown here after Transfer to Medium without Hormones. Small Shoots did not Elongate Normally. Figure 4. A Small Single Shoot was Initiated from Cotyledon Callus Subcultured Three Times in Late 1974. Figure 5. When Cotyledon Callus was Transferred to Medium without Hormones, Shoots Elongated and Produced Normal Appearing Needles. Figure 6. One Shoot was Produced from Needle Callus Subcultured Three Times on Medium Containing Hormones. Figure 7. One Shoot was Produced from Callus on a Stem Explant from a Cotyledon Callus Shoot

rooting medium containing 1-10 mg/liter IBA (indolebutyric acid) in the same fluorescent-tungsten light. However, from the 80-100 attempts, only two shoots rooted after treatment with 10 mg/liter IBA, indicating that agar may be a poor rooting medium. In the first case, rooting occurred after several months on the rooting medium. In the second case, rooting did not occur until the shoot had successively been transferred to medium with 1 mg/liter IBA, medium E-1, 10 mg/liter IBA, and to medium E-1. During this time the shoot produced callus from its base and several new shoots grew from the callus. One vigorous root 2 cm long was initiated from the callus nearly one year after the original shoot was initiated from cotyledon callus.

During this past year we have continued to put out excised embryos and produce cotyledon callus; however, hardly any shoots have been produced from the many established clones. In looking for an explanation, we discovered that all seeds during this past year were sterilized with bleach instead of alcohol. We ran a few tests using the two methods, and found differences in the pattern of shoot initiation directly from cotyledons. We are now testing the hypothesis that bleach sterilization of seeds subsequently inhibits shoot initiation from subcultured cotyledon callus.

Shoots from Needle and Stem Callus

For the past 6-7 years, we have been attempting to produce shoots from subcultured stem callus, initiated from cambial and cortical cells from branches of several conifer species (4,5,16,21). After 2-3 years in culture we observed small, nonfunctional budlike structures on callus of some species (Table I). However, no further shoot organization has taken place in these old cultures, and all except the Douglas-fir callus have been discarded. New cultures of Douglas-fir and loblolly pine are now being used for shoot-initiation studies (5). However,

recent results in our laboratory, and in that of Cheng (9), indicate that the mass production of Douglas-fir may be easier using needle callus than by using stem callus.

A normal seedling was grown from an excised Douglas-fir embryo placed on medium E-1. When the seedling was three months old and 4-5 cm tall, whole needles were excised and placed on medium 10 in 5000 lux continuous fluorescent light at 25°C. From the base of the needles, callus grew and was subcultured three times during four months on medium 10. Eleven days after the third subculture one shoot started to grow from the callus. Figure 6 shows the shoot six weeks later. However, it died without elongating shortly after it was photographed.

In a similar type of experiment, needle and stem explants were prepared from elongating shoots initiated from subcultured cotyledon callus. Some stem segments were placed on medium 19 with 1 mg/liter 2,4-D (2,4-dichlorophenoxyacetic acid). Callus grew rapidly from the explants but was not isolated or subcultured. One vigorous shoot (Fig. 7) grew from the callus of one explant, but when it was isolated from the callus the original explant was not recognizable and we could not determine if the shoot arose from the explant or from callus. We are currently attempting to repeat these results on a larger scale.

Discussion

We have demonstrated propagation methods similar to those reported by Sommer et al. (6,7) for pine species, Campbell and Durzan (8) for white spruce, and Cheng (9) for Douglas-fir. From whole embryos we obtained a few vigorous shoots from the tips of swollen cotyledons using 0.05 mg/liter BAP, but with 0.1 mg/liter we observed various degrees of initiation of small and numerous shoots. Campbell and Durzan (8) obtained maximum frequencies of hypocotyl shoot initiation

using 2 mg/liter BAP and no auxin, but also obtained nearly as good initiation using 2 mg/liter BAP and 0.1 mg/liter NAA (naphthaleneacetic acid). When we used 2 mg/liter BAP and 0.02 mg/liter NOAA (naphthoxyacetic acid), one of five embryos on one dish became completely covered with small budlike structures, each less than 1 mm in length. Unfortunately, the embryo was never transferred and eventually died.

Shoot initiation from Douglas-fir cotyledons apparently was controlled by the level of cytokinin added to the medium. Interestingly, we observed the same relationship in cytokinin control of shoot initiation from aspen callus (22). For both aspen and Douglas-fir, a few vigorous shoots were produced by 0.05 mg/liter BAP, and small multiple shoots were produced with 0.1 mg/liter BAP. For both species, the small shoots did not elongate while on the callus; but for aspen, only the small shoots rooted in high frequencies when excised.

On the other hand, our results with subcultured cotyledon and needle callus were different than expected. Callus from both sources was maintained on medium 10 having 0.1 mg/liter BAP and 5 mg/liter NOAA, and yet the initiation of vigorous shoots was common from cotyledon callus and one shoot was produced from needle callus. This might indicate that either the theory of cytokinin induction of shoots is not valid in callus cultures, or else cytokinin may have been produced endogenously and thus preserved the critically high ratio of cytokinin to auxin. This problem certainly requires a great deal of new information, but perhaps future studies should not be designed to conform strictly to currently popular theories.

Genetic variability was demonstrated in our results with cotyledon callus, but was not surprising because of the variability demonstrated elsewhere by conventional silvicultural and rooting studies. However, by designing

specific nutrient media, someday we may be able to screen seedlings for superior characteristics using aseptic culture techniques.

To date, the state of the art has developed to the point where the clonal propagation of seeds can now become a routine practice, either from cotyledons or from subcultured cotyledon callus, depending on the number of ramets needed. For the propagation of seedlings 3-4 years old or trees 10-20 years old, the most promising method seems to be from needle callus. However, for the most efficient, effective, and economical mass production of conifer trees, the eventual method will most probably have to be from suspension cultures. The progress now being made in several laboratories makes this a realistic goal.

OPTIMIZING THE GROWTH MEDIUM FOR LOBLOLLY PINE CALLUS

Introduction

Several attempts have been made to establish longterm callus cultures of loblolly pine (Pinus taeda L.). About five years ago we established an excellent culture of rapidly growing light-yellow callus in the dark. However, we have had difficulty in maintaining rapid growth of green callus for more than 4-6 months in the light. Loblolly pine callus was initiated three times last year from stem callus and was reported on page 19, Progress Report Three, January 1976. The dates of initiation and sources are listed below:

1. March 1975 Texas Surviving callus from one 3-yr seedling.
2. October 1975 Georgia Ten provenances, one surviving culture.
3. November 1975 Georgia Ten local trees, three surviving cultures.

The problem we have faced in utilizing this callus is that, by the time we have had enough callus to work with, the quality of the callus has suddenly

decreased. In addition, the time of this decrease in quality has differed from one culture to another and could not be anticipated.

All of these cultures were started from one branch per tree of current-year growth, and the 10-15 explants from each branch were distributed among 2-3 different growth media. From the surviving callus initiated on the explants, more callus had to be grown over a period of several months until there were 20-30 dishes of callus. Most cultures did not survive this long, and the ones that did usually started degradation at about the same time that sufficient callus was available. This limited the number and extent of experiments involving new loblolly pine callus, and most of the surviving callus was used in attempting to optimize the growth medium rather than to attempt shoot initiation or start suspension cultures.

For loblolly pine, our objective continues to be the development of a satisfactory nutrient medium that will permit the continuous culture of stem callus in the light, which will eventually produce either shoots from callus cultures or embryoids and plants from liquid suspension cultures.

Literature

Winton (16,23) listed 24 pine species that have been cultured from somatic (nonreproductive) tissue, and Brown and Sommer (24) listed eight references to specifically culturing loblolly pine. Of these eight, Brown (25) obtained callus on the base of shoot apices cultured in Knop's medium, and Konar (14) attempted to initiate callus from stem explants of six conifer species including loblolly pine. However, Konar and Nagmani (3) made no further mention of loblolly pine callus initiated during the original study. All other references listed by Brown and Sommer (24) are to callus produced from roots, parts of seed embryos, or from 1-month-old seedling stem segments.

The first continuous cultures of loblolly pine callus were established by Lawrence and Brown (26) from seed embryos, using Brown and Lawrence (20) medium. Brown and Lawrence (BL) medium is a modification of the nearly universal Murashige and Skoog (27) medium. It does not contain glycine, the sucrose is reduced, and kinetin and 2,4-D (2,4-dichlorophenoxyacetic acid) are added at 0.5 and 5 mg/liter, respectively, as the cytokinin and auxin growth hormones.

Medium 10 (Table II) is a modification of Brown and Lawrence medium, made by replacing kinetin with 0.1 mg/liter BAP (benzylaminopurine) and replacing 2,4-D (2,4-dichlorophenoxyacetic acid) with 5 mg/liter NOAA (naphthoxyacetic acid). Loblolly pine cotyledon callus grows rapidly in the light as light-green callus on medium 10, and in the dark loblolly pine stem callus grows rapidly as light-yellow callus. However, to our knowledge, no one has successfully grown continuous cultures in the light of loblolly pine stem callus. We believe that light-grown stem callus offers a higher probability of shoot propagation than dark-grown callus. We can initiate stem callus easily and it grows rapidly for awhile in the light as light-green callus. However, within a few weeks it starts to develop tan areas and grows slowly as a lower-quality callus for several passages until it finally turns tan or brown and dies.

Methods and Materials

In attempting to optimize a growth medium for loblolly pine, we have tried to make changes in different parts of the chemical and physical environment that we and others believe may influence growth. These include the quality and quantity of light and temperature as well as components of the nutrient medium. A nutrient medium usually consists of inorganic salts, vitamins, sucrose, and growth hormones (cytokinin and auxin). An ideal approach is to run factorial experiments where all possible combinations of two or more factors are used as

treatments, either singly or together. Obviously, this approach requires a large amount of callus, which was not available to us. We therefore had to design factorials, then select small areas to test within the factorials that might offer satisfactory combinations. This modified-factorial approach leaves much to be desired, but was the best we could do with the material available. Our hope was that we could learn enough by making large jumps in factor levels, so that we could go back to promising areas later and make finer adjustments. Some of the factors tested during the past year are as follows:

Inorganic salts:	Calcium at normal and one-half levels Phosphorus at normal and double levels
Vitamins:	With or without nicotinic acid, pyridoxine, thiamine and inositol
Amino acids:	With and without asparagine and tryptophan
Sucrose:	Generally left unchanged at 3%
Cytokinins:	Kinetin or benzylaminopurine at 0, 0.1, 1 mg/liter Isopentenylaminopurine 0.01 mg/liter
Auxins:	NOAA and 2,4-D at 0, 0.5, 1, 5 mg/liter
Light:	Fluorescent light at 2000 or 5000 lux Fluorescent 4000 lux plus tungsten 500 lux Continuous or 16 hr light and 8 hr dark
Temperature:	Lab temperature 22-25°C Constant 25°C light or 27°C dark 24°C light and 18°C dark for 16/8 hr cycle

Results

For initiating loblolly pine callus, growth was fastest and greenest for 1-2 weeks on medium 10 in constant fluorescent light at 5000 lux. However, subcultured callus grew better in tungsten and fluorescent light. Calcium at one-half strength (220 mg/liter) was better than at full strength, and a double amount of phosphorus (340 mg/liter) was better than normal. The addition of

tryptophan also stimulated growth. Using a medium-10 base, growth was better with 2,4-D than with NOAA at 5 mg/liter. However, when vitamins, inositol and asparagine were omitted, 1 mg/liter 2,4-D was better than 5 mg/liter.

In addition to testing variations of medium 10, we also tried media used by Harvey (28), Schenk and Hildebrandt (29), Sommer, Brown and Kormanik (7), and Hare (unpublished). We tried again the original Brown and Lawrence medium (20), the basis of medium 10, but have still found no other medium that does as well as our own modifications of medium 10, which we designate as medium LV-10.

At present we have callus cultures growing in several environments on several media from all surviving 1975 callus initiations. Most, however, are growing slowly and are light-yellow green or yellow brown. The best callus is yellow green and growing rapidly in the lab, on medium LV-10 in fluorescent-tungsten light for 16 hours per day alternating with 8 hours of darkness. Medium LV-10 contains low calcium, high phosphorus, tryptophan, and 10 mg/liter NOAA.

Future Plans

We are now in the process of trying to put together all of the best features of the best media found during several studies. We will test medium LV-10 with different levels of 2,4-D, and also try different levels and durations of fluorescent-tungsten light. Meanwhile, we are using some new callus to test shoot-initiation treatments.

EMBRYOID INITIATION IN DOUGLAS-FIR CALLUS CULTURES

Introduction

We have tried for several years to produce shoots from old Douglas-fir stem callus grown in light for 6-7 years. We feel that this callus is now too

old to produce shoots unless it can be rejuvenated in some manner. One indication that rejuvenation has occurred may be the initiation of E-cells or small embryoids in the callus. Another indication that an optimum hormone balance has been achieved may not be the absence of both growth or death in callus, but rather the continuing no-growth of healthy green callus. This hypothesis is derived from our experience with aspen callus. When we found shoots, they were generally from callus pieces having little or no growth, but remaining white or sometimes slightly tan. We hope that finding nutrient or environmental factors that will initiate embyroids in Douglas-fir callus, or conditions that will prolong healthy callus without death or growth, may help establish a base from which we can attempt shoot initiation from newly-initiated callus of Douglas-fir and loblolly pine.

Methods and Results

Several different experiments were run this past six months, which are outlined below. The results are included after each experiment.

Experiment 1. Callus KL17 and KL13-MO were grown on medium 10 having 0.05 mg/liter 2,4-D and either 0.2 or 2 mg/liter BAP. Some of the media were also made without any ammonium, but having the same amount of nitrogen with added potassium nitrate. The innoculated plates were grown in various light and temperature combinations and checked after a month or so.

The best dark-green callus was soft and uniformly undifferentiated, and grew from the KL17 callus on medium 3 with 0.2 BAP and ammonium in 5000 lux of continuous light. On the other hand, the most dividing E-cells were in the cultures of callus KL13-MO on the same medium, but in callus transferred from the same high light to 2000 lux fluorescent light for 16 hours at 24°C, alternating with 8-hour dark at 18°C.

Experiment 2. Callus DL3 and KL17 were placed in two replicated plates per treatment of medium 17 made with 0, 0.0001, 0.001, 0.01, 0.05, 0.1, 0.5, 1, 5 and 10 mg/liter 2,4-D. All callus originally grew in the lab under continuous 5000 lux fluorescent light at 23-26°C, and all treatments were transferred to an incubator with continuous 5000 lux light at 24°C.

At the end of the first 6 weeks, all cultures were still alive but the extreme concentrations of 2,4-D appeared detrimental. DL3 callus, grown on stock medium 17 containing 2,4-D, turned yellow/brown at 5 and 10 mg/liter and maintained healthy green tissue at all lower concentrations. However, KL17 callus from medium 3 with 1 mg/liter NOAA was less sensitive to the higher concentrations but remained green and viable at only the midconcentrations, 0.05-1.0. The lasting and cumulative effects of 2,4-D were apparent. All callus was treated at this time with liquid medium having 20 mg/liter BAP, and after one week one replication of all treatments was transferred to medium without hormones. The other replication was left on the same test medium on which it had been treated with BAP. After another month all callus KL17 was dying or dead and all DL3 callus remained green except for tissue originally put out on the lowest 2,4-D test media. No shoots were observed nor were cytological examinations made. The surviving DL3 callus, representing tissue originally grown on test media with 0.5-10 mg/liter 2,4-D, and subsequently treated with BAP and transferred after one week to medium without hormones, has just been transferred again to fresh medium without hormones.

Experiment 3. Callus DL3 and KL17, grown on medium 3 and 10, were placed on medium 19 made with 0.001M thiourea (76 mg/liter) alone, or with thiourea and 2 mg/liter BAP, or with thiourea and 100 mg/liter inositol, 0.4 mg/liter thiamine and 0.1 mg/liter BAP. Most plates were placed in 2000 lux fluorescent

light for 16 hours at 24°C, alternating with 8 hour dark at 18°C. Some treatments were also placed in 5000 lux continuous light in the lab.

The treatments in the lab grew faster, but died sooner than callus in the incubator with variable light and temperature. Callus DL3 survived longer than KL17, and callus without BAP survived longer than with BAP. Surviving callus in the incubator grew slower but lasted for three months before it was discarded.

Experiment 4. Four callus tissues were placed on 28 variations of medium 10. The tissues were KL17 on medium 10, KL13-MO on medium 3, new Douglas-fir callus on medium 10, and cotyledon callus number 65 on medium 10. The variations of medium 10 were from a factorial design of 0, 0.01, 0.05, 0.1, 0.5, 1 and 5 mg/liter BAP combined with all levels of 0, 0.01, 0.1 and 1 mg/liter NOAA. All plates were placed in the variable incubator described in Experiment 3.

After one month, all surviving plates were examined and described. The data are not given, but a synopsis is given for each callus tissue source.

KL17-10: Green and moderate growth at 0 BAP/0.1 NOAA and 1 NOAA, as well as at 1 NOAA at BAP 0-1. Best at 1 each BAP/NOAA.

KL13-MO-3: Best green growth at 1:1 BAP/NOAA, at 0.01 each and 1 each. However, good green growth also at 0.01 and 1 NOAA at BAP 0-1.

New DF-10: Best at 1 NOAA and 0.1-5 BAP. Dead without hormones.

CC DF-10: Like new DF. Best dark green at 1 NOAA and 0.1-5 BAP. Medium 3 has 0.1 BAP and 1 NOAA.

Our plans are now to again examine the callus after two months and also cytologically look for embryoids in all surviving cultures.

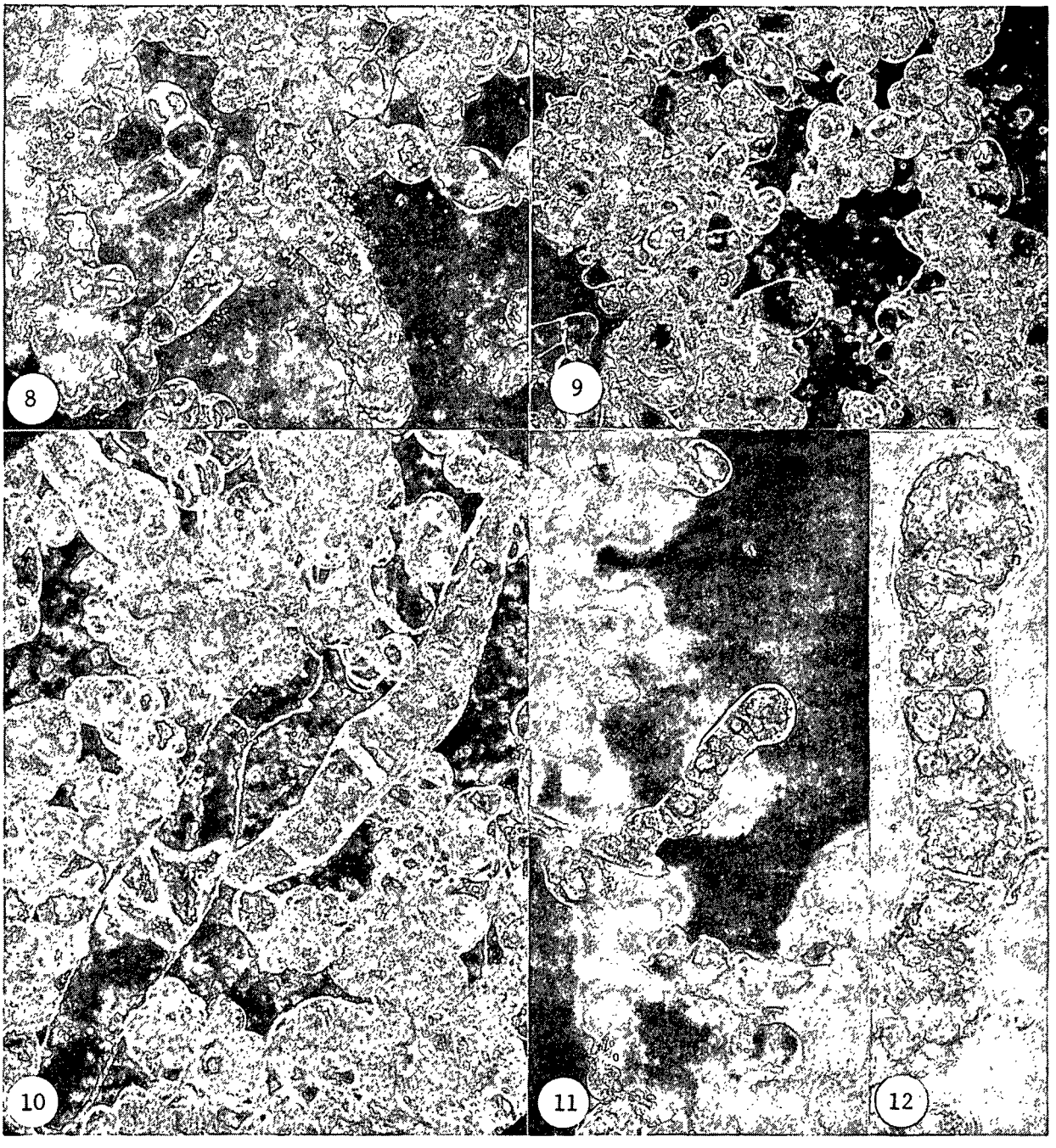
Experiment 5. Cold treatments were given to DL3 callus on medium 17 by placing several plates in the refrigerator at 3°C and bringing out one plate after 1, 4, 6 and 8 days to the lab light at 5000 lux. Two controls were left uncovered in the lab and one plate was covered with foil for 8 days.

After one month, there were no significant differences in callus appearance between treatments, except that the callus left in the lab covered with foil was darker in color than the others. All other callus pieces are still light-yellow green with darker centers.

Experiment 6. Many plates of callus have been moved from one light source to another without any chemical treatment. The purpose was to test only the difference in light quantity or day length, as well as the temperature difference associated with 16-hour photoperiods.

After several months of shifting plates around, the general results indicate that callus should not be moved from low light to higher light, but that more cell differentiation occurs if the callus is always moved from high to low light and from continuous to 16-hour light. For both KL17-17 and KL13-MO-3, callus growing in continuous 5000 lux fluorescent light in the lab is already producing E-cells and some embryoids under those conditions. However, if both are moved to 5000 lux for 16 hours at 24°C, alternating with dark for 8 hours at 18°C, occasional medium-sized and rare large embryoids can be observed after 1-2 weeks.

Figures 8 and 9 show stock cultures of callus KL13-MO and KL17, respectively, in the lab under continuous 5000 lux light. Figures 10-12 show the same callus, after 15 days in the incubator with alternating light and temperature, with embryoids composed of cells with large vacuoles (KL13) or with small vacuoles (KL13-MO).



Figures 8-12. Callus Before and After Transfer from Lab to Incubator Light

Fig. 8. Stock Callus KL13-MO on Medium 3 in Continuous Light

Fig. 9. Stock Callus KL17 on Medium 17 in Continuous Light

Fig. 10. Large Embryoids of KL13-MO 15 Days after Transfer to Incubator with Variable Light and Temperature

Fig. 11. Large Embryoid of KL17 15 Days after Transfer to Same Variable Incubator. Fig. 1-4 all 140X

Fig. 12. Enlargement of Fig. 4 Embryoid. 350X

We are now using these callus cultures in attempts to enlarge embryoids in suspension cultures. We also plan to try more elaborate sequences of changes, particularly of taking callus from high to very low light. We also believe that an interrupted photoperiod is best for cell differentiation, but not as favorable to the maintenance of a uniformly undifferentiated stock culture.

Future Plans

We have been working on many factors that may increase cell differentiation and organogenesis in stem callus, and the problem now is to try to separate out the most important ones for further study. Much more biochemical work can be done on stem callus, and there may even be a possibility of producing shoots from old stem callus if it is rejuvenated or from newly-initiated stem callus under the optimum environmental conditions. However, for the next six months we may de-emphasize shoot-initiation experiments with old or new stem callus, and concentrate more on attempting to initiate shoots from needle callus.

ENZYME INVESTIGATIONS

Introduction

During the past few months our research on enzymes of conifer tissue has come to focus on oxidases and related activities. In particular, research has centered on peroxidases, catalase, indoleacetic acid (IAA) oxidases, phenol oxidases, superoxide dismutases, and polyamine oxidases. Our earlier data obtained in comparative analyses for the peroxidases suggested that they might bear some relationship to organizational processes and would warrant further scrutiny. The other enzymes listed have rather direct metabolic relationships with the peroxidases. The desirability of more investigation was further enhanced by positive feedback responses which could be related to peroxidase activity. It may be recalled that

polyamine additions had given improved callus greening and chloroplast structure and also had promoted longevity in perturbation research, so there was that additional incentive to look at polyamine oxidase activity.

Methods

Most of the enzyme investigations were conducted by disk electrophoresis followed by on-gel staining for enzyme activity, but there were some wet assays. Both standard anionic and cationic systems (30) were used in the electrophoresis reported here; unless noted otherwise, the cationic system was that containing β-alanine at pH 4.3. Catalase and peroxidase activity were both determined using diaminobenzidine (DAB) as adapted from Gregory and Fridovich (31). In the absence of exogenous horseradish peroxidase, the procedures of this reference yield peroxidase patterns essentially the same as obtained with conventional substrates like benzidine or guaiacol (see previous reports). Catalase activity in a peroxidase band can be picked out by using the horseradish peroxidase (Worthington) and running several levels of enzyme (i.e., callus extract).

Phenol and polyphenol oxidase were detected by standard procedures (32). Location of IAA oxidase bands was done according to Hoyle (33) while the superoxide dismutase on-gel staining procedure was that of Bohnenkamp and Weser (34). Wet assays for catalase were performed according to Beers and Sizer (35) and for peroxidase according to Cohen (36). Wet assays for amine or polyamine oxidase activity were based upon the assumption that such activity would lead to hydrogen peroxide production which was then used to oxidize guaiacol in the presence of added horseradish peroxidase (37). Samples consisted of 2.0 ml of 0.1M buffer [citrate at pH 4.5, phosphate at pH 7.2 or morpholinopropane sulfonate (MOPS) at pH 7.0], 0.1 ml of amine or polyamine solution (3 mg/ml solutions in the appropriate buffer of tryptamine, spermidine, spermine, or putrescine as hydrochlorides), 0.1 ml of

horseradish peroxidase (1 mg/ml in the appropriate buffer), 0.1 ml guaiacol (25 mM in the appropriate buffer), and 0.5 ml of callus extract (prepared by extraction of an acetone powder of callus with one of the above buffers followed by "desalting" of the extract on a Sephadex G-25 column equilibrated with the same buffer). Controls were run containing buffer in place of extract and also some lacking only substrate.

Results

Progress Report Three contained several electrophoretic distribution patterns for isoperoxidases of both Douglas-fir and loblolly pine extracts, and other patterns were prevented in Progress Report Two. By now we have in hand a substantial number of distribution patterns for several enzymes from many samples of stem callus, cotyledon callus, and organized tissue. Variables represented in these samples include age since initiation of callus, age since subculture of callus, composition of growth medium on which callus was grown, clone, etc. Representative data will be presented here on an enzyme-by-enzyme basis.

Peroxidases and Catalase

Isoperoxidase electrophoretic banding patterns are given in Fig. 13 for Douglas-fir stem callus initiated in the spring of 1975 and for Douglas-fir seedlings (hypocotyls and cotyledons only). The outstanding difference noted in the unorganized versus organized tissue comparison in the figure is in the size of the last peak to the right. More important, perhaps, note the size of this peak relative to the sizes of the other peaks in each case. That the last peak to the right has catalase as well as apparent peroxidase activity has been demonstrated repeatedly. This is shown in Fig. 14 where, under certain experimental conditions (31), this peak appears double and dominates the pattern. Figure 15 shows that the catalase peak (as measured by its apparent peroxidase activity)

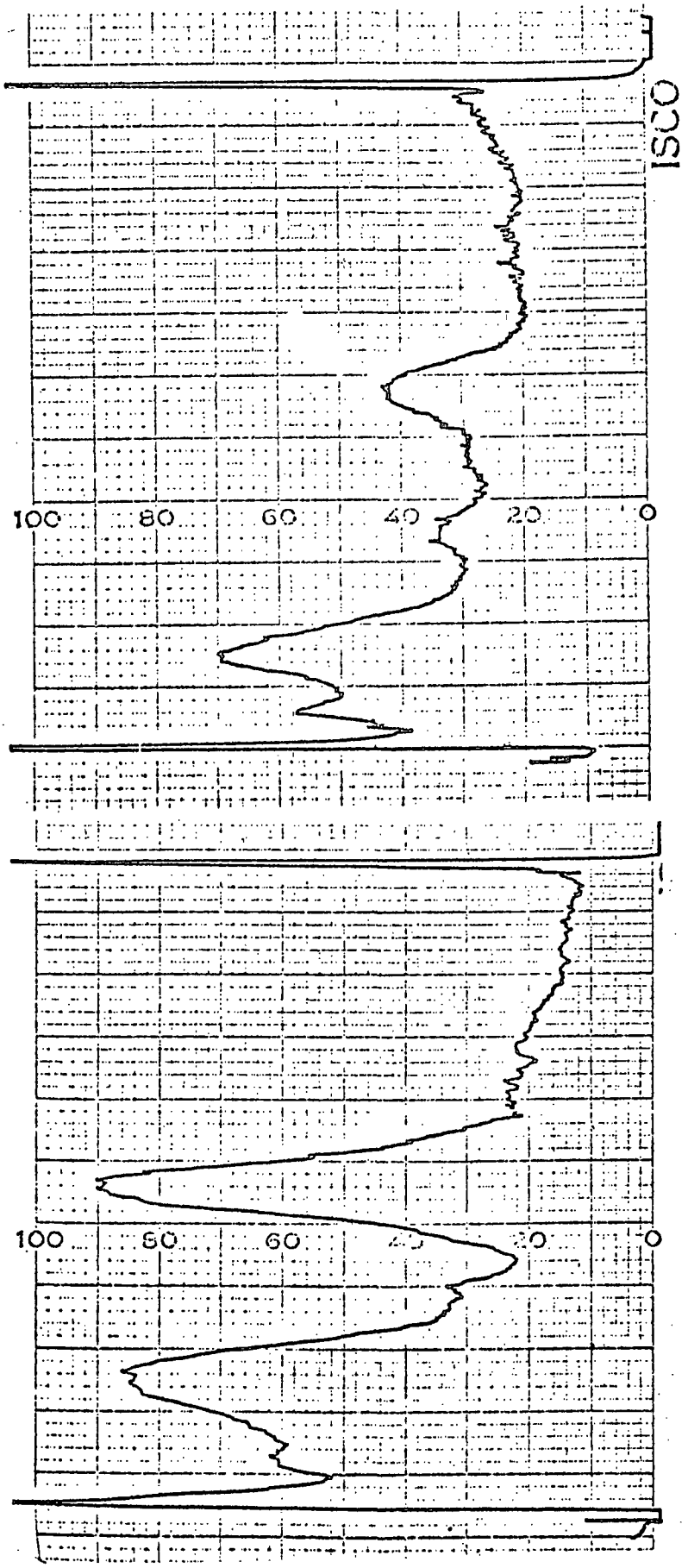


Figure 13. Peroxidase-Catalase Distribution After Anionic Electrophoresis: (a) Douglas-fir Stem Callus Assayed 29 Days After Subculture 9 Times on Medium 10, (b) Hypocotyls and Cotyledons of Douglas-fir Seedlings

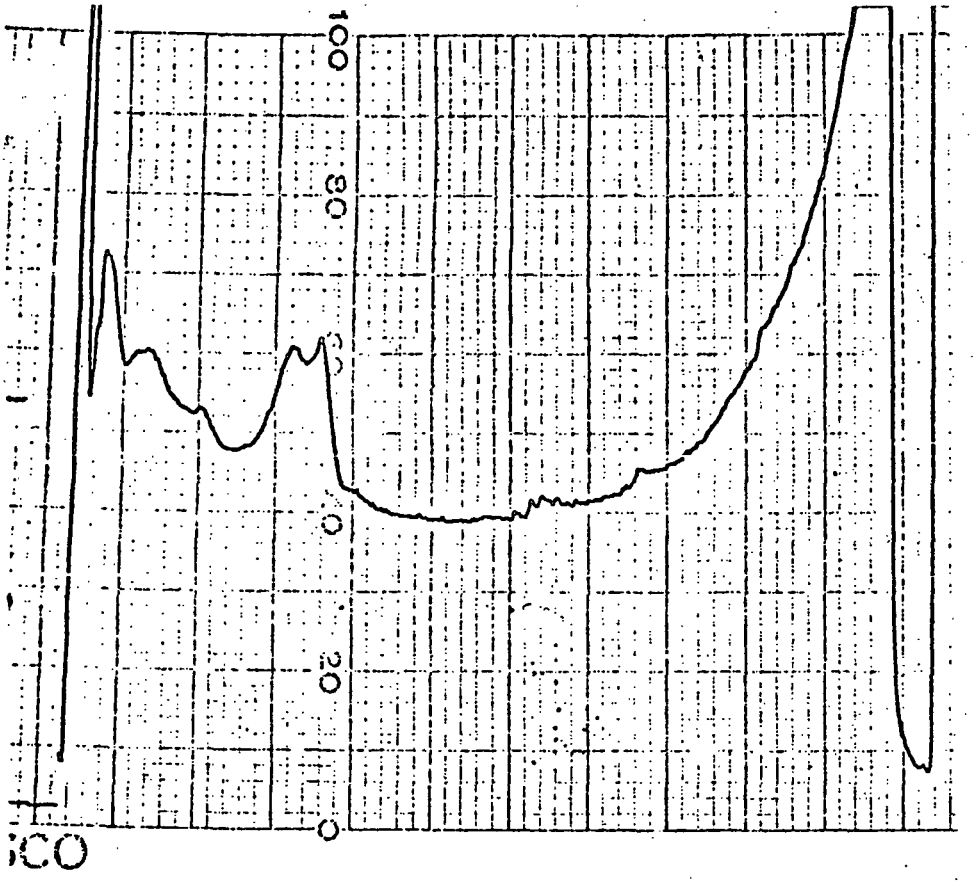


Figure 14. The Catalase Double Peak as a Result of Negative Staining

develops more rapidly than the other peroxidase peaks. The general picture given in Fig. 13 is fairly typical of comparisons of isoperoxidase and catalase banding patterns between callus and organized tissue although there is some "wobble" due to factors like age, medium, etc., as shown in Fig. 16 to 18. Figure 16 shows some of the variation in these banding patterns that are encountered in Douglas-fir stem callus under various conditions. Progressing from Fig. 16a-16c one can see a gradual drop in the catalase peak which appears to be mostly a function of age since subculture. By comparing Fig. 16b, 16d, and 16g, one is viewing mostly the effects of callus culture on three different media. In the series 16d-16f can be seen the effect of transferring from one medium to another as the pattern gradually approaches that of 16b. The sample for pattern 16h had a very complex

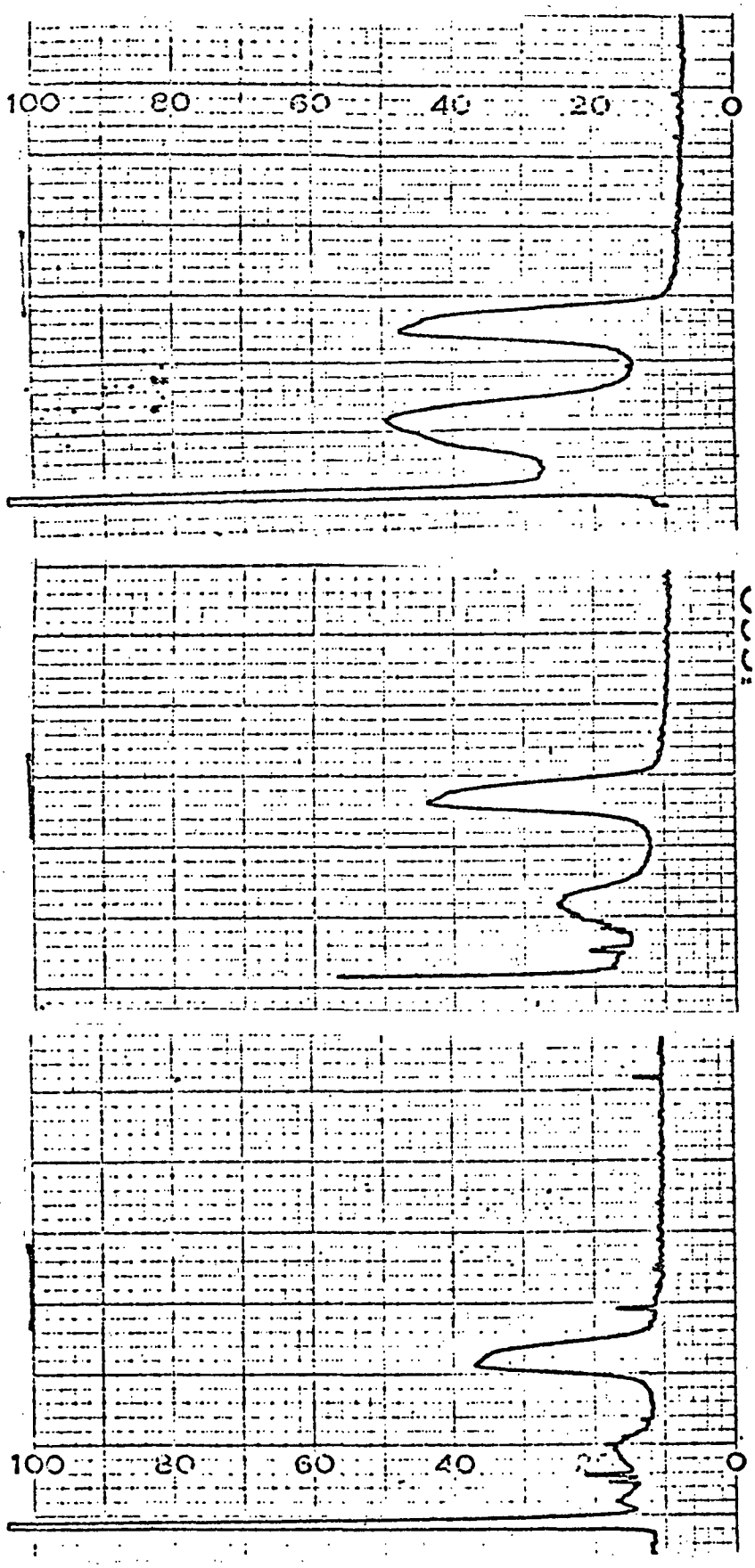


Figure 15. The Development of Peroxidase-Catalase Bands with Time Following Anionic Electrophoresis. The Peak to the Right is Catalase at Each Time

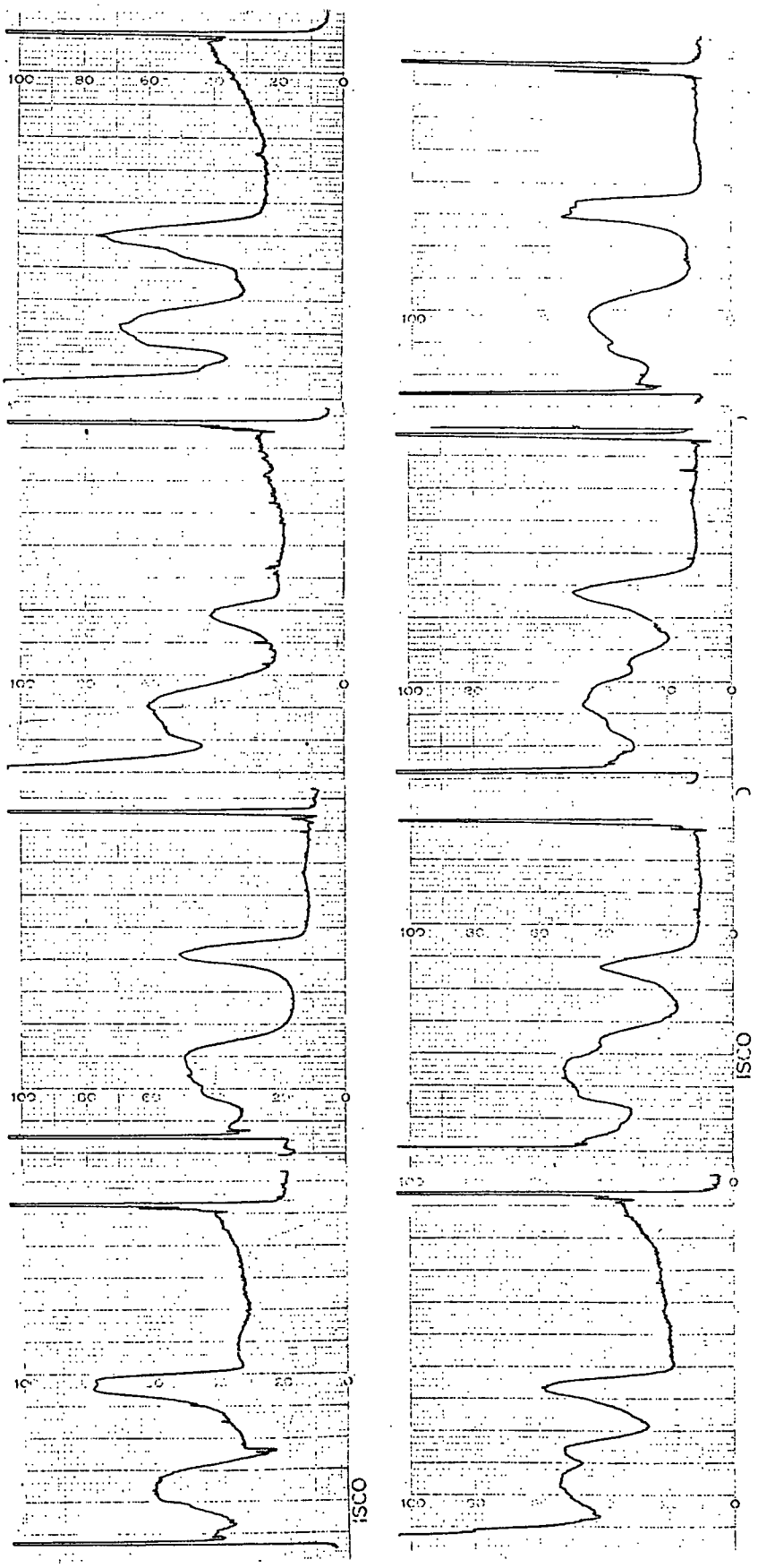


Figure 16. Douglas-fir Peroxidase-Catalase Distribution After Anionic Electrophoresis as a Function of Culture Conditions: (a) Stem Callus Source Used in Most Enzyme Investigations Assayed 16 Days After Subculture 59 Times on Medium 17-8, (b) Stem Callus as in (a) Assayed 21 Days After Subculture 59 Times on Medium 17-8, (c) Stem Callus as in (a) Assayed 79 Days After Subculture 57 Times on Medium 17-8, (d) Stem Callus Assayed 30 Days After Subculture 60 Times on Medium 3, (e) Stem Callus as in (d) Assayed 44 Days After Subculture 57 Times on Medium 3 Then Twice on Medium 17-8, (f) Stem Callus as in (d) Assayed 17 Days After Subculture 57 Times on Medium 3 Then Three Times on Medium 17-8, (g) Stem Callus Assayed 24 Days After Subculture 61 Times on Medium 10, (h) Stem Callus Assayed 21 Days After Subculture 32 Times on Medium 17-8 but Prior to that had Been on Medium 17-8 Containing Lysine and Still Earlier had Been in Suspension Culture for a Time

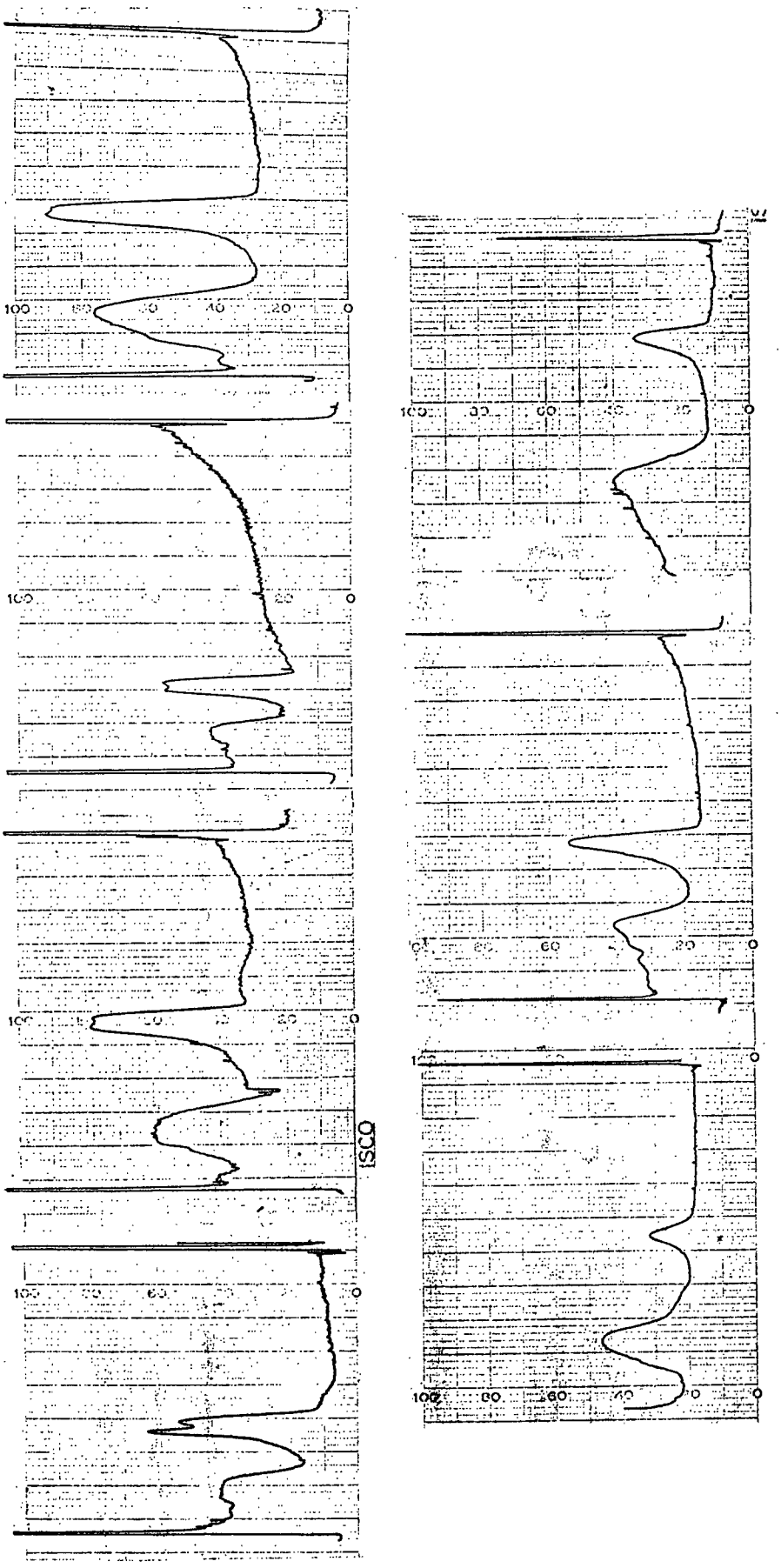


Figure 17. Douglas-fir Stem Callus Peroxidase-Catalase Distribution After Anionic Electrophoresis as a Function of Number of Subcultures and Time of Assay Since Last Subculture on Medium 17-8: (a) Assayed 32 Days after the 57th Subculture, (b) Assayed 16 Days after the 59th Subculture, (c) Assayed 17 Days after the 61st Subculture, (d) Assayed 24 Days after the 61st Subculture, (e) Assayed 29 Days After the 61st Subculture, (f) Assayed 14 Days after the 63rd Subculture, (g) Assayed 27 Days after the 63rd Subculture

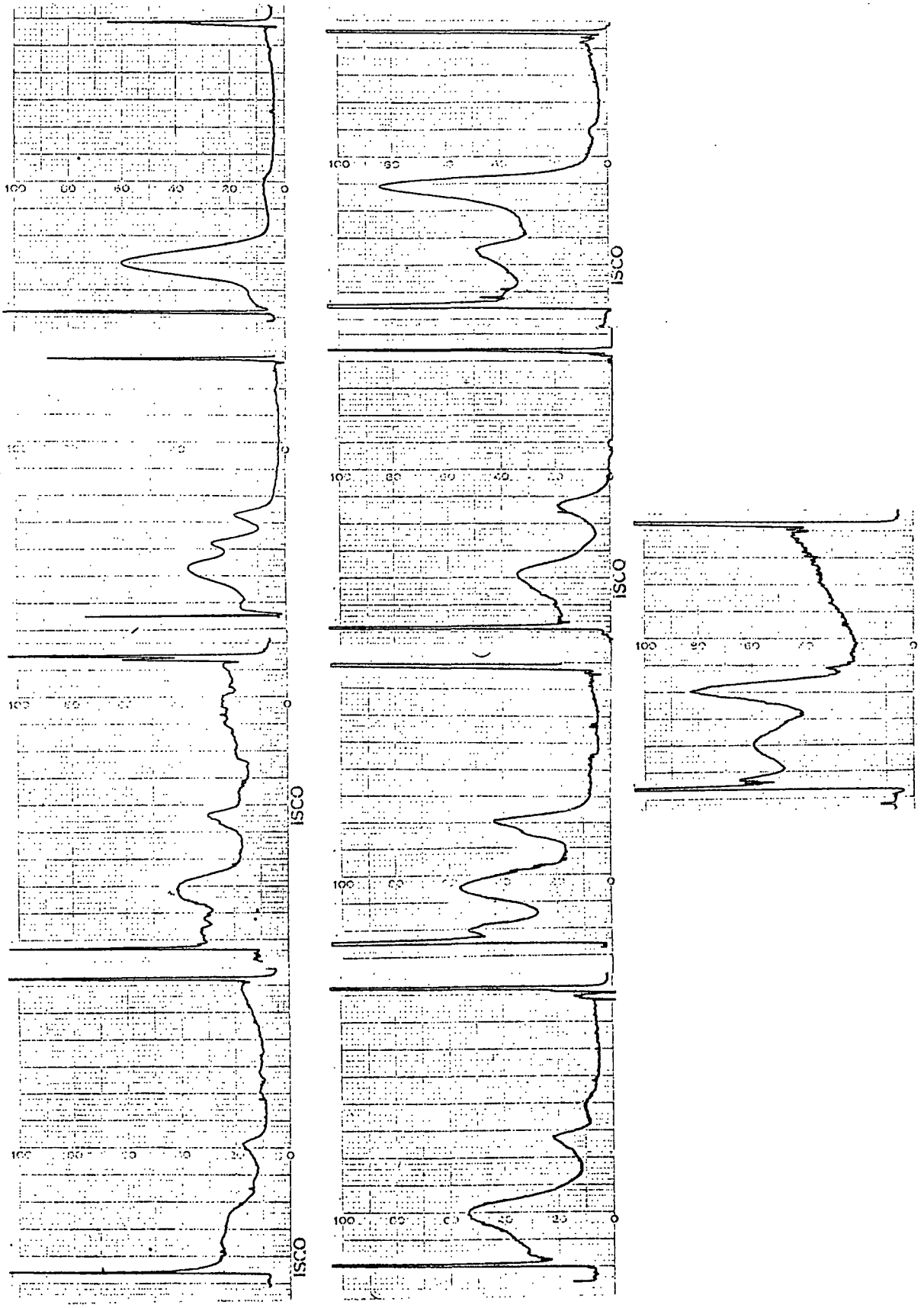

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Figure 18. Douglas-fir Cotyledon Callus Peroxidase-Catalase Distribution After Anionic Electrophoresis as a Function of Clone, Number of Subcultures, and Time of Assay Since Last Subculture on Medium 10: (a) Clone 44 Assayed 59 Days After the 8th Subculture, (b) Clone 44 Assayed 64 Days After the 8th Subculture, (c) Clone 44 Assayed 26 Days After the 10th Subculture, (d) Clone 64 Assayed 57 Days After the 5th Subculture, (e) Clone 69 Assayed 57 Days After the 3rd Subculture, (f) Clone 66 Assayed 36 Days After the 5th Subculture, (g) Clone 34 Assayed 19 Days After the 16th Subculture, (h) Clone 58 Assayed 20 Days After the 7th Subculture, (i) Clone 67 Assayed 9 Days After the 5th Subculture

history. The main differences between the sample used for Fig. 16g and that used for Fig. 13a were number of subcultures and genetics; however, the patterns are really very similar indicating that neither of these factors was very important in at least this case. The banding pattern as a function of number of subcultures versus time of assay after the last subculture is examined further in Fig. 17. The general impression is that the latter is the more important parameter with catalase activity rising after subculture and then gradually declining with time. This impression is reinforced by the patterns for cotyledon callus given in Fig. 18 although there also appear to be some real clonal differences.

In April of this year a visit was made to the laboratory of Dr. M. C. Hoyle of the Forest Service in Durham, New Hampshire. Dr. Hoyle was kind enough to subject some of our freeze-dried samples of organized and unorganized tissue to his isoelectric focusing technique. The results of one of his runs is presented in Fig. 19 with his permission and our gratitude. It can be seen that our stem callus extract is characterized by extremely cationic and anionic species with

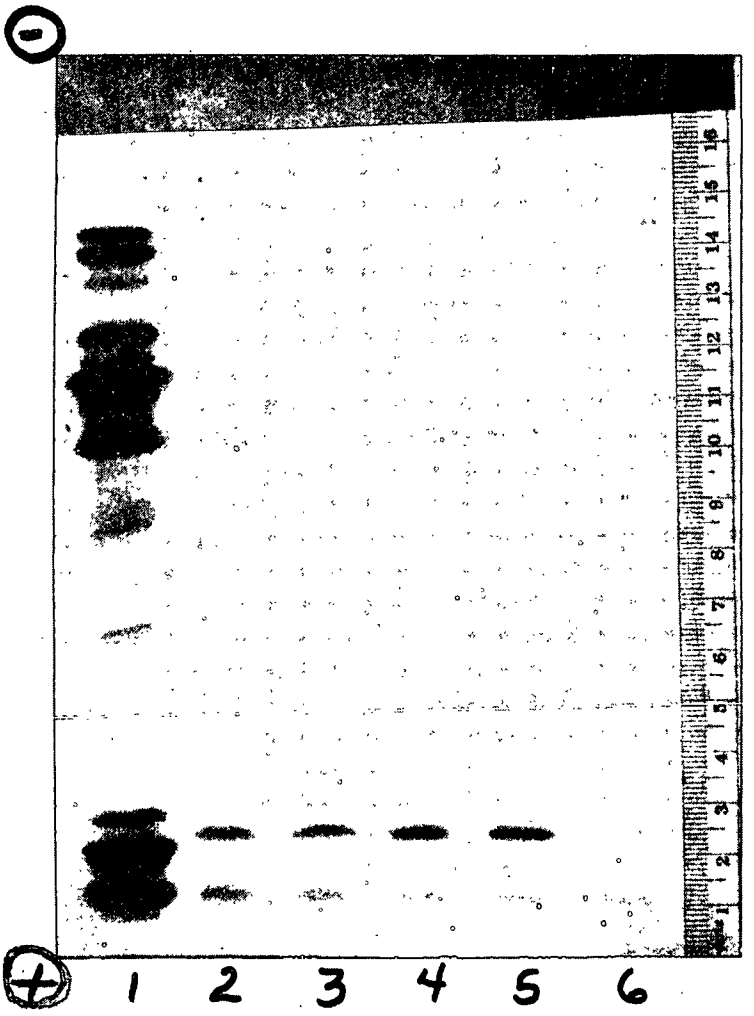
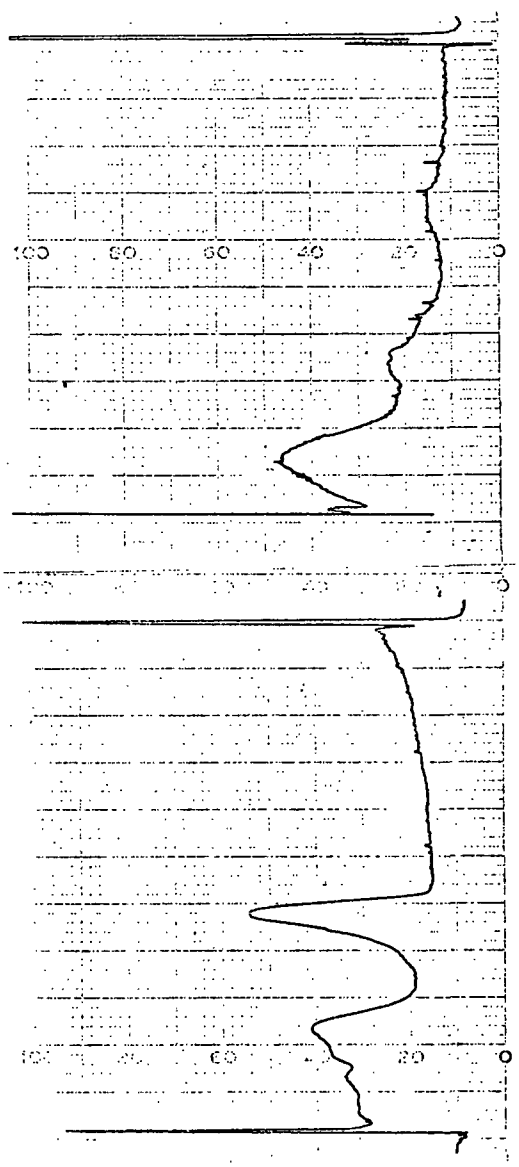


Figure 19. Douglas-fir Stem Callus and Seedling Extracts Isoelectric Focused and Stained for Peroxidase (Courtesy Dr. M. C. Hoyle). Samples were Loaded in the Center of the Layer and Components Proceed to Either the Anode (Bottom) or Cathode (Top). The Samples are Left to Right: Horseradish Peroxidase Standard, Callus Extract in Water (2), Callus Extract in Buffer (2), and Seedling Extract in Buffer

little of intermediate character while our organized issue extract is more complex but still not approaching the complexity of the horseradish peroxidase control. This data provides a reference point for our disk electrophoresis data. While isoelectric focusing provides greater resolution, the fact that bands from our extracts are not too numerous nor bunched together by that technique suggests that our disk electrophoresis data collected to date may still be quite representative of the true situation.

The differences in isoperoxidase and catalase electrophoretic banding with organizational state were examined further as shown in Fig. 20 (anionic system) and Fig. 21 (cationic system). For this purpose, Douglas-fir seedlings from the greenhouse were divided into three sections (true stem and needles, cotyledons, and hypocotyls with roots). Acetone powder extracts of each of these sections were prepared, assayed separately and compared with Douglas-fir stem callus extracts as shown in the figures. The major difference between the unorganized callus tissue and the various seedling sections noticeable in Fig. 20 still is the relatively large size of the catalase peak relative to other peaks in the case of callus only. One can also pick out other differences between organized and unorganized tissue and among the three sections of the seedling such as the sharp, fast-moving peak of the hypocotyl and root section extract which is only a low broad hump in the other two sections. Also there appears to be a low mobility peak in the true stem and needle extract only. In the cationic systems (Fig. 21) one can see first of all that the running pH makes a substantial difference in the first two patterns (a versus b). The callus extract traces are dominated by a low mobility peak and quite reproducible except for the large absorption near the bottom of the gel in Fig. 21c. The various organized sections of the seedlings are on the other hand dominated by a peak of intermediate mobility and the cotyledons



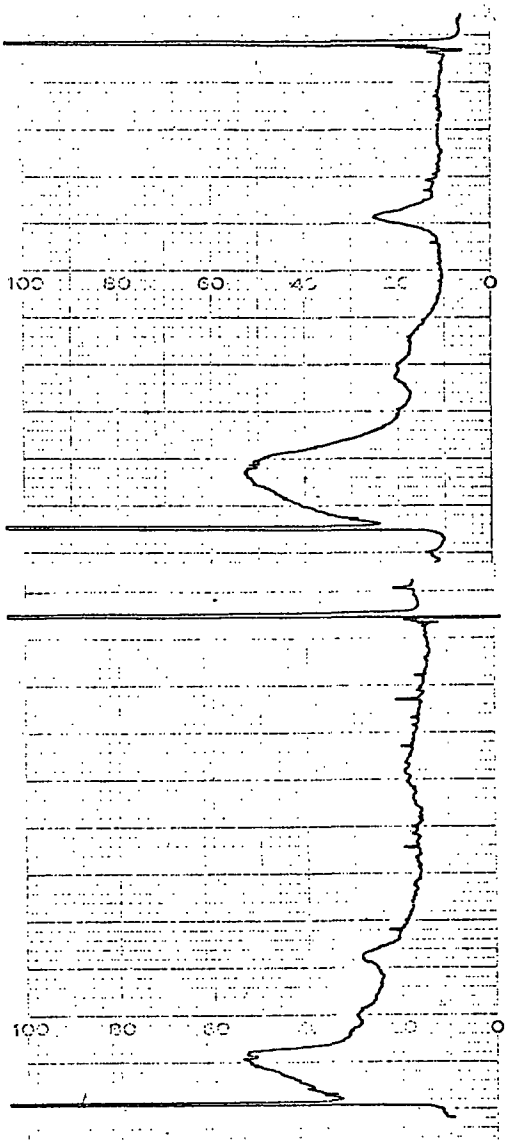


Figure 20. Douglas-fir Peroxidase-Catalase Distribution After Anionic Electrophoresis as a Function of Organizational State: (a) Stem Callus Assayed 14 Days After the 63rd Subculture on Medium 17-8, (b) True Stems and Needles, (c) Cotyledons and (d) Hypocotyls and Roots

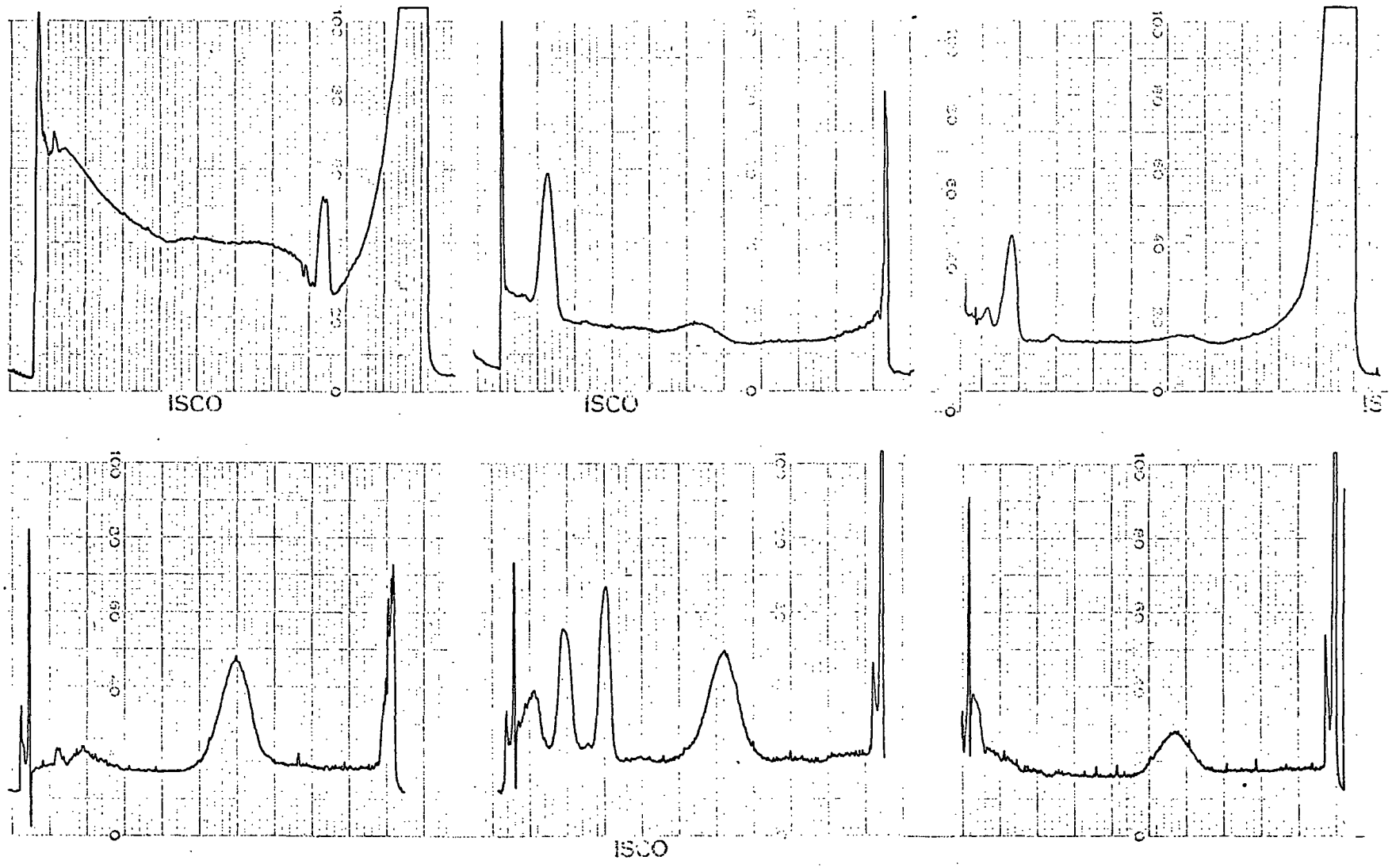


Figure 21. Douglas-fir Peroxidase-Catalase Distribution After Cationic Electrophoresis as a Function of Organizational State and Other Conditions: (a) Stem Callus Assayed at pH 2.3 35 Days After the 61st Subculture on Medium 17-8, (b) Stem Callus Assayed at pH 4.3 21 Days After the 62nd Subculture on Medium 17-8, (c) Stem Callus Assayed at pH 4.3 15 Days After the 63rd Subculture on Medium 17-8, (d) True Stems and Needles at pH 4.3, (e) Cotyledons at pH 4.3, (f) Hypocotyls and Roots at pH 4.3

exhibit extra peaks that may be very active versions of very minor peaks from the callus extract. Similar studies have been conducted by other researchers with other plants (e.g., 38-40), and some of the comparisons that can be made with their data are very intriguing.

IAA Oxidases

IAA oxidases may be isozymes of peroxidase, i.e., isoperoxidases with some specificity for IAA. This relationship may be similar to the observation that catalases exhibit peroxidase activity but not necessarily. There is in fact evidence in the literature (33) that in some plants all isoperoxidases have IAA oxidase activity, but there is no unaminous accord on this point. It has been difficult to demonstrate any IAA oxidase activity in extracts of Douglas-fir stem callus by on-gel staining after either anionic or cationic electrophoresis. Evidence for distinct band formation is a little better for organized tissue (Fig. 22) following anionic electrophoresis. The weak apparent activity found in some stem callus extracts would seem to have too low a pH optimum (<4) to be very functional in vivo (however, recall that pH's of suspension cultures rapidly drop to that level) while that from organized tissue seems to have a broader range extending at least up to pH 6. More input is needed from cationic runs and from wet assays before we will have a very clear picture of IAA oxidase in these tissues. It is certainly not true in our experience that all isoperoxidases have IAA oxidase activity although this may relate to preparative techniques and the retention or loss of heme prosthetic groups and may yet be contradicted by better staining techniques or the wet assay of eluted bands.

Superoxide Dismutases

These enzymes which catalyze reactions that destroy the superoxide ion have become quite common in the biochemical literature in just the past couple of

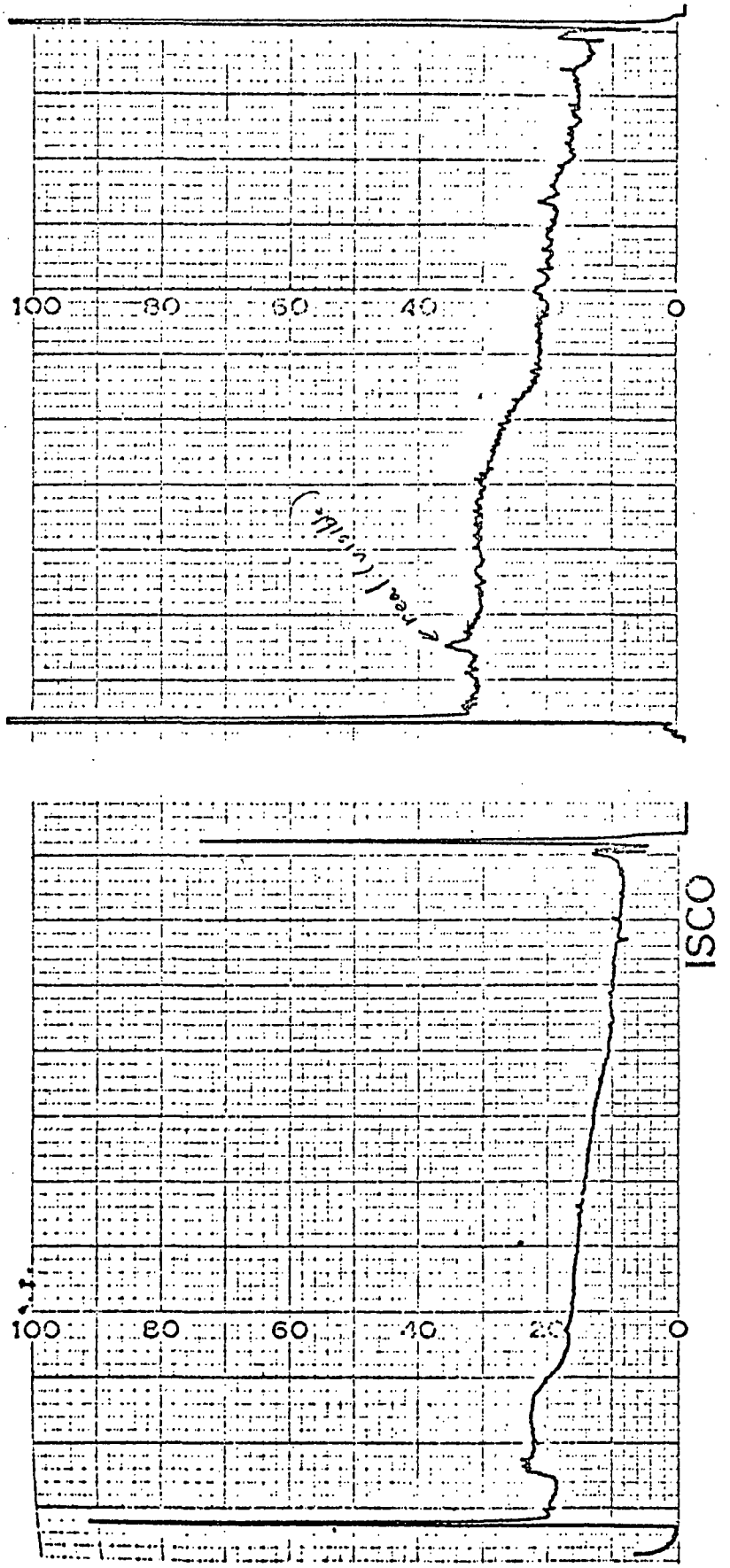


Figure 22. IAA Oxidase Distribution After Anionic Electrophoresis as a Function of Organizational State: (a) Douglas-fir Stem Callus, (b) Douglas-fir Seedlings

years. Since they are involved with excited states of oxygen and with hydrogen peroxide, they have been of some interest in this project for some time. The recent publication that showed peroxidases capable of generating superoxide ions (41) made some experimental work imperative. Figures 23 and 24 show the on-gel scans for superoxide dismutase as a function of organizational state following anionic and cationic electrophoresis, respectively. In the anionic system (Fig. 23) one can see that the banding pattern of this enzyme also appears to be changing with time from subculture. The banding pattern for the cotyledon extract (Fig. 23d) seems to incorporate features from both Fig. 23a and 23b while the stems and needles extract (Fig. 23c) has no low mobility peak and the hypocotyl-root section is generally weak in this enzyme. Note: this is a negative staining procedure so that the peaks in Fig. 23 and 24 are inverted. In the cationic system (Fig. 24) more variability in the banding pattern from stem callus extracts is encountered, apparently as a function of number of subcultures and time from subculture, and the banding patterns from organized tissues are different but not sharply defined. More work with this enzyme is needed, but it also appears to fluctuate with plant development. Matkovics (42) has found this enzyme as well as peroxidase and catalase to exhibit periodicity during bean plant development. He points out that while superoxide dismutase can convert superoxide to hydrogen peroxide and oxygen, enzymes like phenol oxidases (below) can do just the opposite.

Phenol Oxidases and Polyphenol Oxidases

Banding patterns for these enzymes following anionic electrophoresis have been presented previously (i.e., Fig. 16 and 16a of Progress Report Three) when some doubt about their being separate enzymes was expressed. This is an activity which appears to a small extent even without adding an exogenous artificial substrate. Callus tissue extracts are characterized by large peaks of low and

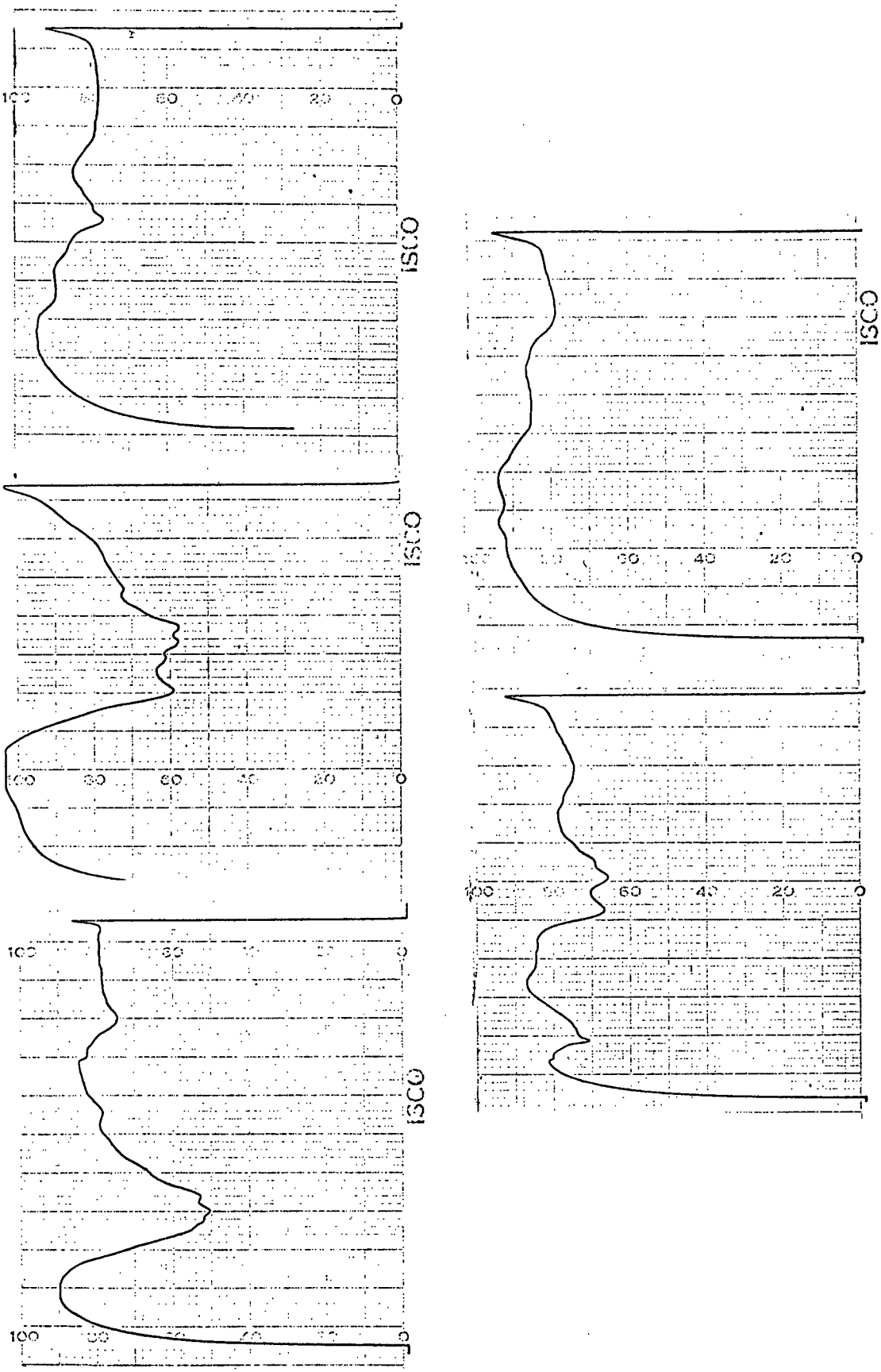


Figure 23. Douglas-fir Superoxide Dismutase Distribution After Anionic Electrophoresis as a Function of Organizational State and Other Conditions: (a) Stem Callus Assayed 14 Days After the 63rd Subculture on Medium 17-8, (b) Stem Callus Assayed 25 Days After the 63rd Subculture on Medium 17-8, (c) True Stems and Needles, (d) Cotyledons, (e) Hypocotyls and Roots

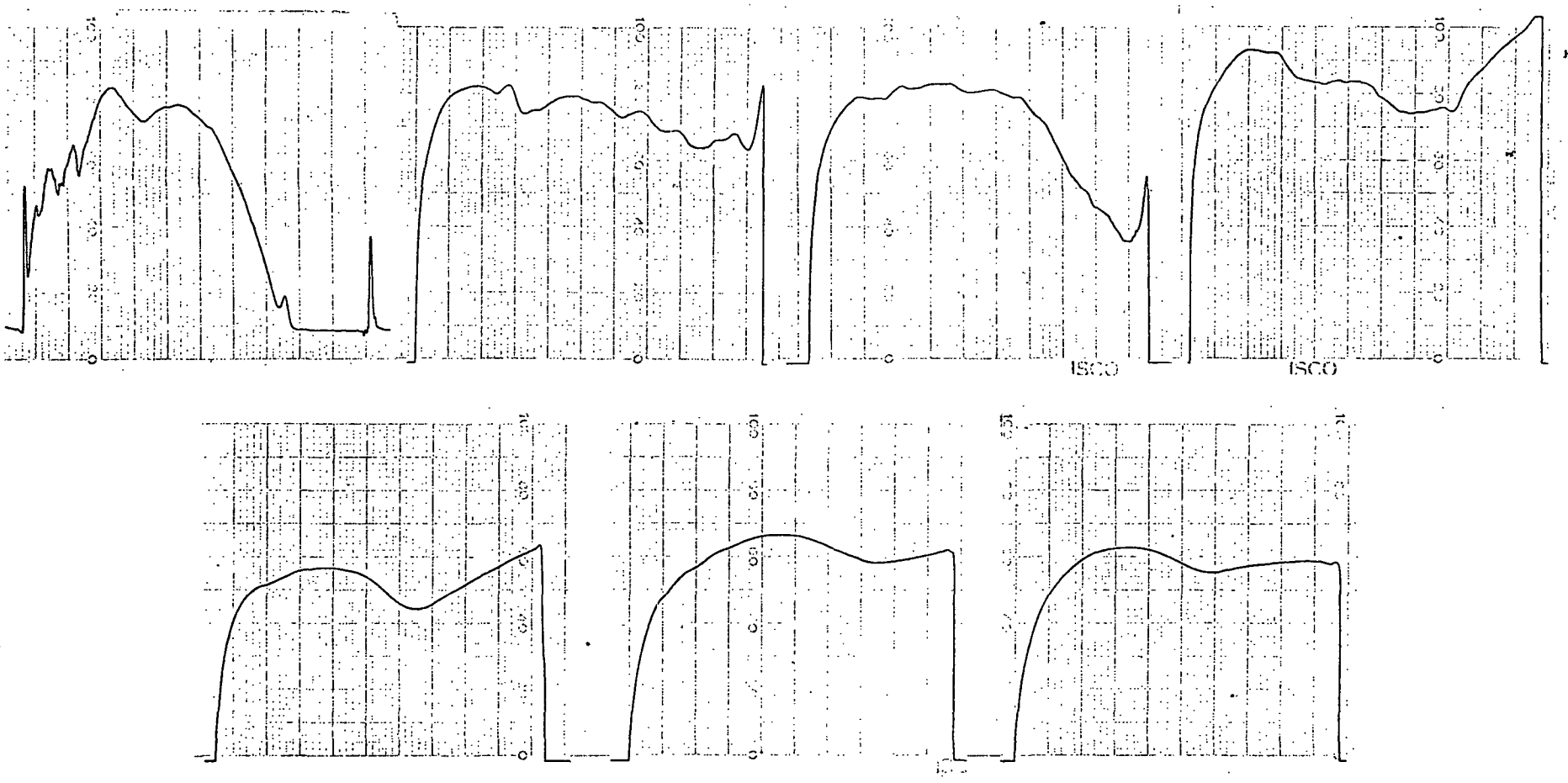


Figure 24. Douglas-fir Superoxide Dismutase Distribution After Cationic Electrophoresis as a Function of Organizational State and Other Conditions: (a) Stem Callus Assayed at pH 2.3 35 Days After the 61st Subculture on Medium 17-8, (b) Stem Callus Assayed at pH 4.3 21 Days After the 62nd Subculture on Medium 17-8, (c) Stem Callus Assayed at pH 4.3 15 Days After the 63rd Subculture on Medium 17-8, (d) Stem Callus Assayed at pH 4.3 29 Days After the 63rd Subculture on Medium 17-8, (e) True Stems and Needles at pH 4.3, (f) Cotyledons at pH 4.3, (g) Hypocotyls and Roots at pH 4.3

intermediate mobility while the organized tissue (Fig. 25) gives greater prominence to a faster moving peak barely visible in banding patterns of callus extracts. In cationic electrophoresis (Fig. 26) one again sees a large effect of running pH. Comparisons between callus extract (Fig. 26b) and the various seedling sections (Fig. 26c, 26d, and 26e) again show differences, mostly in the relative sizes of the peaks. Cotyledon extracts (Fig. 26d) again appear to have "extra" peaks.

DAB Oxidase

Exactly what this enzyme is or what its relationship is to the other enzymes that have been discussed above remains to be determined. As shown in Fig. 27, this is a band of oxidative activity which develops with the DAB substrate in the absence of exogenous hydrogen peroxide. While it develops more slowly than do peaks of DAB oxidation products in the presence of hydrogen peroxide, it does become quite large in time and causes interference in wet assays of peroxidase in unfractionated extracts. It appears that this peak may indicate that there is in situ generation of hydrogen peroxide. It has shown great sensitivity to the presence of mercaptoethanol which may be useful in further investigations.

Polyamine Oxidases

Monoamine and polyamine oxidase activities were examined in extracts of Douglas-fir stem callus, cotyledon callus, stems and needles. Some data were also obtained for loblolly pine needle extracts. Extracts of organized Douglas-fir tissue show little or no activity unless one processes the stems and needles separately. It is then found that stem extracts are fairly active, especially against spermine and spermidine at neutral pH. Loblolly pine needle extracts appear to be even more active under these conditions (no data yet available for loblolly pine stem extracts). In contrast to this, extracts of both cotyledon callus and stem callus of Douglas-fir were inactive at neutral pH although the

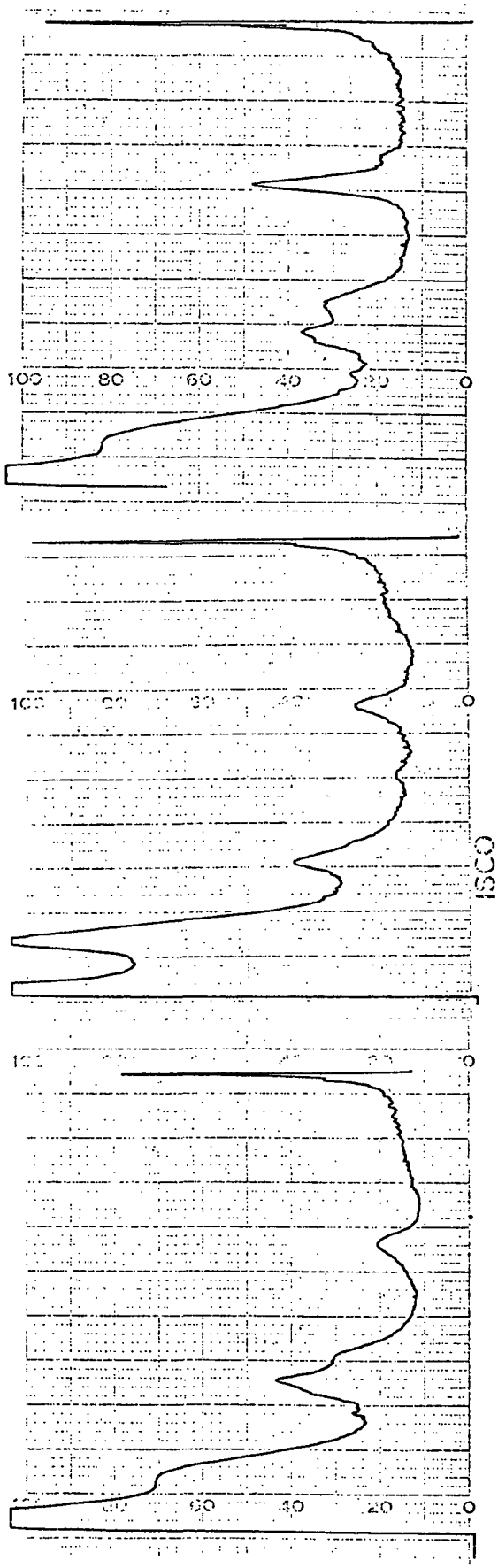


Figure 25. Distribution of Phenol Oxidase in Douglas-fir Organized Tissue After Anionic Electrophoresis: (a) True Stems and Needles, (b) Cotyledons, (c) Hypocotyls and Roots

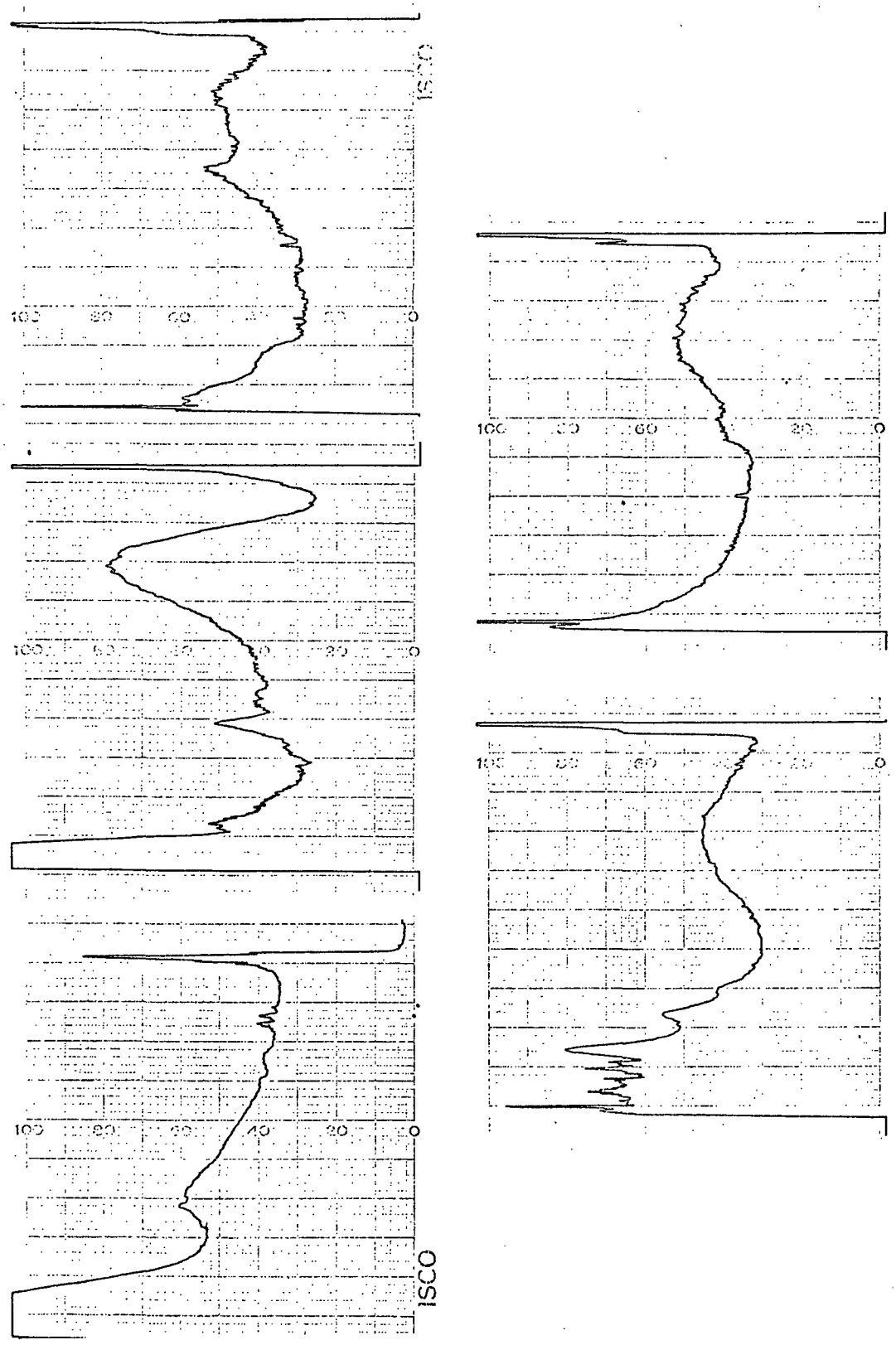


Figure 26. Douglas-fir Phenol Oxidase Distribution After Cationic Electrophoresis as a Function of Organizational State and Other Conditions: (a) Stem Callus Assayed at pH 2.3 35 Days After the 61st Subculture on Medium 17-8, (b) Stem Callus Assayed at pH 4.3 21 Days After the 62nd Subculture on Medium 17-8, (c) True Stems and Needles at pH 4.3, (d) Cotyledons at pH 4.3, (e) Hypocotyls and Roots at pH 4.3

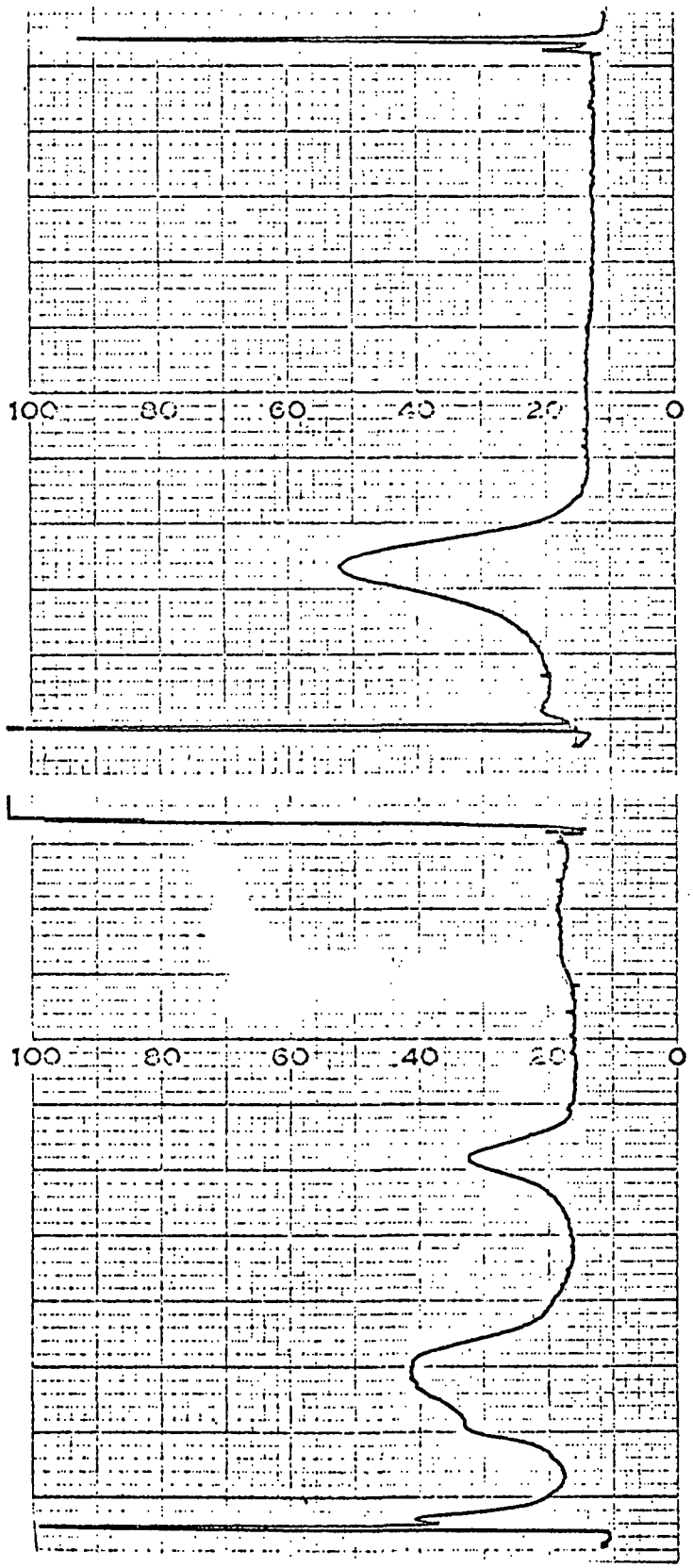


Figure 27. Peroxidase-"DAB Oxidase" Distribution After Anionic Electrophoresis: (a) Peroxidase with Hydrogen Peroxide Present, (b) "DAB Oxidase" without Exogenous Hydrogen Peroxide Present. Overnight Development

cotyledon callus extracts were active at pH 4.5. Extracts of all tissues mentioned except the Douglas-fir needles and stem callus also contain material (probably protein) which is precipitated by spermine and spermidine. At this time it appears that Douglas-fir stem callus cannot oxidize polyamines. This is interesting because one of the products of polyamine oxidase action should be hydrogen peroxide, the oxidizing agent in peroxidase activity and an effective agent in feedback experiments. Furthermore, the biosynthetic formation of polyamines should proceed from arginine, possibly via ornithine, both of which are also compounds of current interest (see thin-layer chromatography section).

Wet Assays of Catalase and Peroxidase

Some attempts have been made to obtain quantitative data on these enzymes by running wet assays with unfractionated extracts that have been excluded from Sephadex G-25. In the case of catalase these assays have been somewhat successful, and results on an equivalent fresh weight basis are given for callus and various organized tissues in Fig. 28. The actual fresh weight equivalents present in samples during assay in each case were: stem callus 230 mg, stems and needles 100 mg, hypocotyls and roots 64 mg, and cotyledons 66 mg. I should be borne in mind that protein makes up a much larger portion of the fresh weight in the cases of stem callus and cotyledons than it does in the cases of the true somatic tissues. Nevertheless, catalase activity has not been measurable in true stems, needles, hypocotyls and roots of Douglas-fir seedlings employing fresh weight equivalents such as listed above; conceivably, it might be found by using much greater amounts of starting material. It seems notable that on essentially the same basis the seedling cotyledons, a specialized organized tissue, exhibit considerable catalase activity. Certainly, on a fresh weight basis, the catalase activity in callus tissue appears to be a much more important factor to be reckoned with than in true somatic tissue where its very existence is doubtful.

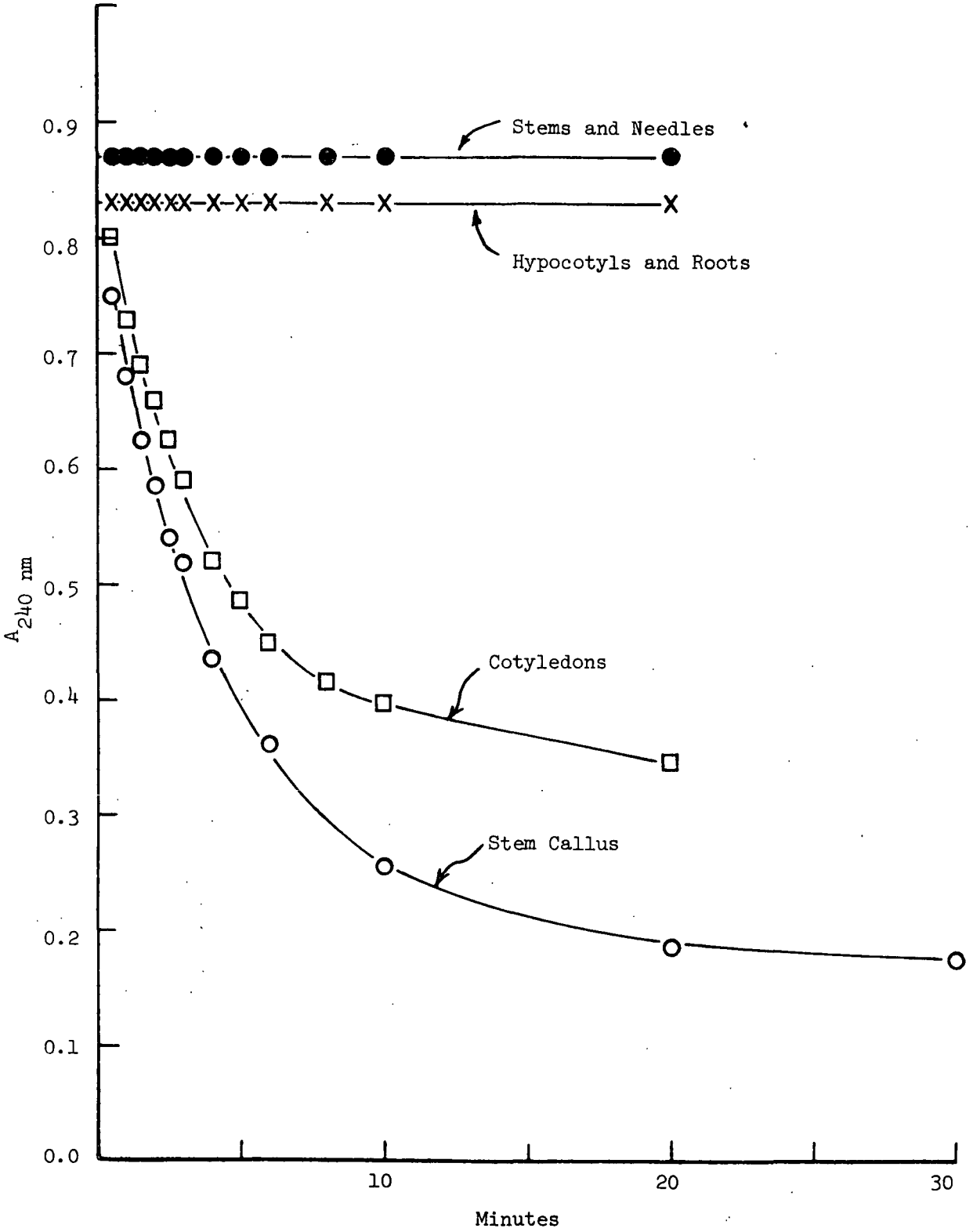


Figure 28. Wet Assay of Douglas-fir Catalase in 230 mg (Fresh Weight) of Various Tissues

In contrast to catalase, at least some peroxidase activity is detectable in all tissues examined (Fig. 29 — results again on an equivalent fresh weight basis). Furthermore, particularly in the case of organized tissue extracts, the peroxidase activity tends to increase as extracts are allowed to stand at room temperature; catalase activity shows the opposite trend. In time progress curves for peroxidase activity for extracts of stem callus, slope reversal occurs within a few minutes and seems to coincide timewise with the decline in slope of the catalase activity time progress curves. The reversal is known to be due to color development in the reference cuvette (which lacks exogenous hydrogen peroxide but does contain extract) which is initially outpaced by the reaction in the sample cuvette to which hydrogen peroxide is added at zero time. This phenomenon appears to involve DAB oxidase (see earlier section), but the relationship is not clear at this time.

THIN-LAYER CHROMATOGRAPHY OF TISSUE EXTRACTS

Introduction

TLC has been used from the beginning of this project as the principal tool in comparative analyses for low molecular weight constituents. This type of activity has tapered off lately, not because of completion but because metabolic studies have taken precedence in recent months. Gelman's ITLC glass fiber sheets, type SA, were used in all TLC reported in this section.

Results

Further TLC of indoles from Douglas-fir cotyledon callus and organized tissue was conducted. The results were very similar to those reported for Douglas-fir stem callus in Progress Report Three. All three types of tissue usually yield only two indole-reactive spots in a standard procedure, one of the spots being at

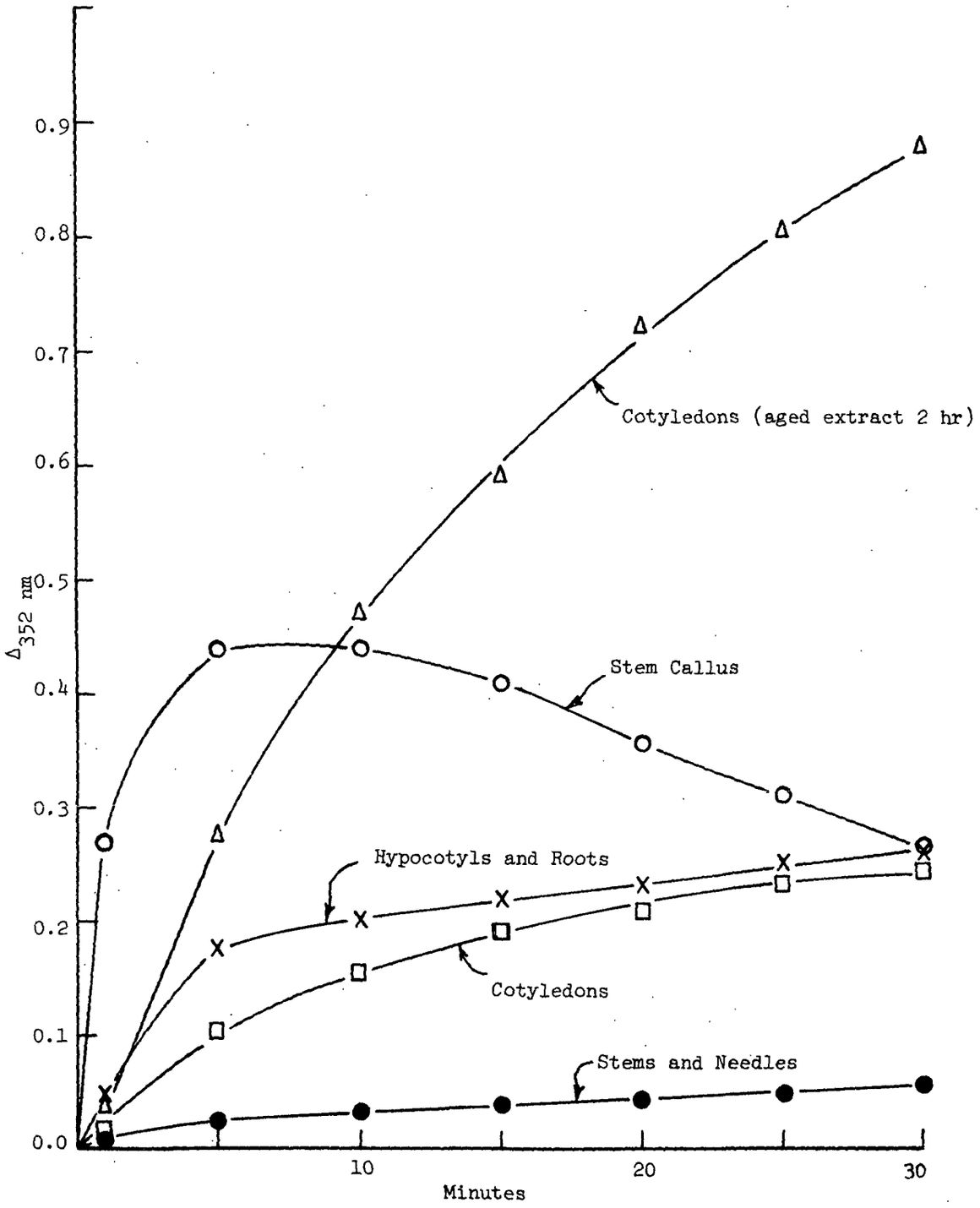


Figure 29. Wet Assay of Douglas-fir Peroxidase in 230 mg (Fresh Weight) of Various Tissues

the origin and associated with polysaccharide and other macromolecules. The origin spot gives the strongest indole reaction with extracts of cotyledon callus. Although some of the associated polysaccharide can be broken down by pectinase, such degradation does not liberate the indole-reactive material as a detectable spot with increased mobility. Aqueous callus extracts have been found to contain considerable amounts of pectic substances; on one occasion a gel formed which could be destroyed by pectinase. The inclusion of pectinase during aqueous extractions improves sedimentability of acetone powder residues but this has not become a routine procedure because there are some drawbacks to having pectinase in the preparation in later assay stages.

The observation during TLC analyses that caused the greatest stir was that our previous reports of large amounts of lysine accompanying massive amounts of arginine in extracts of organized tissue are in error. At least most of the material misidentified as lysine appears to be ornithine (Fig. 30). In solvent systems previously used for amino acid TLC these two compounds had essentially identical Rf's and were not resolved — which is not surprising since they differ in structure by a single methylene group. Because ornithine can be formed from arginine by one-step enzymatic hydrolysis theoretically, its presence rather than lysine sheds new light on the metabolic picture.

NITROGEN SOURCES

Introduction

In conjunction with the thiourea feedback research (see below), it became of concern that stem callus might be producing nitrite from nitrate in the process of nitrogen assimilation. Since nitrite may also inhibit catalase as does thiourea (43), it was thought that the effective concentration of catalase inhibitor

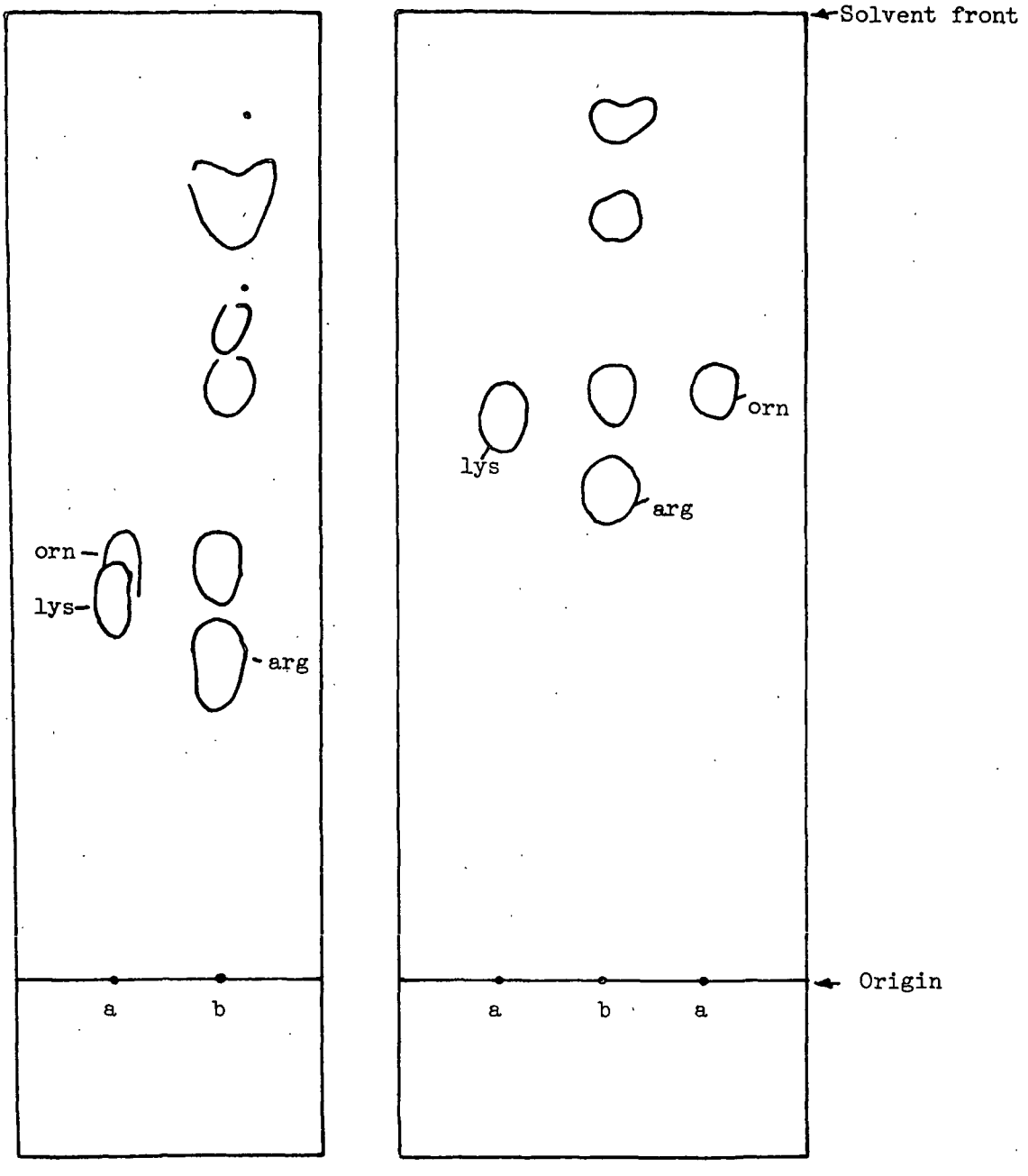


Figure 30. Resolution of Ornithine and Lysine by TLC. The Chromatogram on the Right was Developed Twice in the Solvent ($MeOH/NH_4OH$, 3/liter). a = Standards, b = Seedling Extract

might not be that of thiourea alone. A check on nitrite formation in Douglas-fir stem callus resulted in questions and more tests.

Results

Some stem callus that had been growing on an ammonium nitrate medium for two months was tested for nitrate, nitrite and nitrate reductase. All tests were negative. However, the agar on which the callus was growing gave a strong nitrate test but no test for nitrite nor for ammonia. It was tentatively concluded that stem callus was using only the ammonium portion of the nitrogen supply and that this could perhaps partially account for earlier observations of medium acidification during growth of suspension cells. Further analyses and attempts to grow cells on only ammonium or only nitrate ions as the nitrogen source indicated that nitrate may be used but at a very slow rate and that ammonium had to be kept to a low level to avoid toxicity. There have been recent attempts to use other nitrogen sources such as arginine and urea. It is notable that in the early 1960's Pharis found urea to be the best nitrogen fertilizer for field-grown loblolly pine (44).

GROWTH AND ORGANIZATION OF SUSPENSION CULTURES

ADDITION OF AUXIN TO SUSPENSION CULTURES OF DOUGLAS-FIR

Introduction

In Progress Report Three, page 50, January 1976, the development of basic medium 26-10 for suspension cultures was reported. This is a simplified medium with one-half-normal inorganic salts, 5.6 mg/liter iron, 3.5% sucrose, and a ratio of 400:4000 ammonium nitrate to potassium nitrate (Table VIII). Using this liquid medium we have obtained high frequencies of small embryoids composed of several cells, with occasional large embryoids with up to one hundred or more cells. We used a soft dark-green callus of Douglas-fir growing on medium 3 containing both auxin and cytokinin. We were able to increase the frequencies of large embryoids slightly by centrifuging or by giving the cells short exposures to high levels of cytokinin, but we could not induce the embryoids to continue organizing into true embryos that would grow into plants.

In this study, instead of exposing cells to cytokinin, we added auxin at different levels in order to study its effect on both growth and on embryoid formation.

Methods and Materials

To liquid medium 26-10 was added the auxin 2,4-D (2,4-dichlorophenoxy-acetic acid) at levels of 0, 0.0001, 0.001, 0.01, 0.05, 0.1, 0.5, 1, 5, 10 mg/liter. Ten ml of medium were added to each of one test tube per treatment, and four sets of treatments were prepared for testing four different callus lines. All callus originated from the same piece initiated in 1969 (21), but has been grown on different media for the past several years. The KL designation indicates that the callus at one time was transferred to suspension culture, then returned to agar

medium. The MO callus received a mesityl oxide treatment about a year ago and normally produces a low level of embryoids in the callus. Table IV shows the callus source and the suspension conditions during the experiment.

TABLE IV

CALLUS SOURCES AND ENVIRONMENTAL CONDITIONS FOR SUSPENSIONS

	Source	Suspension Environment
1.	DL3 on medium 17[a] 5000 lux fluor 24 hr, lab yellow-green callus	Fast drum (20 rpm) 1 week, then to slow drum (1 rpm) in same light
2.	KL17 on medium 3[a] same light dark-green callus	Slow drum in same light
3.	KL13-MO on medium 3 same light dark-green callus	Slow drum in same light
4.	KL13-MO on medium 3 same light in incubator light 16 hr, dark 8 hr, 25°C dark-green callus	Slow drum in same light

[a]Mediums 3 and 17 are shown on page 13, Table 1, Progress Report Two.

Each suspension was examined cytologically 1, 5, 8, 11, 14, 19, and 42 days after the start of the experiment. Subjective estimates were recorded of the relative number of cells either having a few or many chloroplasts. Frequencies of cells with light or dark deposits of tanninlike material, or cells having small vacuoles (E-cells) were also recorded, as well as the presence and size of embryoids.

Results

Callus DL3 is normally grown on simplified medium 17 containing 0.5 mg/liter 2,4-D, and apparently some of the 2,4-D auxin was carried over from the agar to the liquid medium. A few embryoids were found in treatments with the

lower levels of 2,4-D, but none were found when 2,4-D exceeded 0.01 mg/liter. With higher amounts of 2,4-D in the suspension, the callus became greener at 0.1-1 mg/liter, then decreased in greenness at 5 and 10 mg/liter. For DL3 callus, 2,4-D did not stimulate the formation of embryoids.

For all KL callus sources from medium 3, however, not only were a few E-cells and embryoids present in the callus cultures, but they continued to form after the callus was placed in the suspension cultures. After 2-3 weeks, significant differences were observed among similar treatments for the three KL sources. More new embryoids formed from the KL-MO callus cells, particularly from the callus cells that had been growing in the incubator before transfer to liquid.

There seemed to be about a nine-day cell cycle, during which the cells remained fairly unchanged, followed by division of a large percentage of the cells within the same day or so. In past work with cell suspensions, most cells usually reverted to large-vacuole stages during division, and E-cell formation started again after each division. In some of the 2,4-D treatments, however, E-cells seemed to remain E-cells during division and clumps of E-cells simply enlarged.

In the future, we may find that embryoids must be transferred to agar medium or to a different liquid medium after nine days, to permit the embryoids to continue development at a slower pace than in the original suspension. Cell division is necessary for embryoid development, but at a slow enough rate to allow E-cells to continue to aggregate. Theoretically, auxin causes cells to enlarge rapidly and become vacuolated, whereas cytokinin causes cell division without cell enlargement. According to this hypothesis, embryoid development should occur with high cytokinin and no auxin, which is the theory that has guided our research for so long. Here, however, we found that in some cases auxin without cytokinin caused embryoid development. As yet, we cannot explain this hormone action.

Generally, with the KL13 callus, as the level of 2,4-D increased from 0 to 0.01 mg/liter, there was an increase in cell size and the number of small embryoids, with a corresponding decrease in green chloroplasts per cell. At 0.05 mg/liter 2,4-D, there was no differentiation and the cells were uniformly dark green and formed the best suspension of well-dispersed cells, even for up to 42 days when the experiment was terminated. From 0.1 to 1 mg/liter, there was an increase in green cells, E-cells, and embryoids. Then at 5 and 10 mg/liter the trend reversed.

Discussion

Liquid medium 26-10 was specifically designed to produce E-cells and embryoids in suspension cultures from KL13 callus grown on agar medium 3, rather than for DL3 callus grown on medium 17. This may explain why more cell differentiation was observed in suspensions of KL callus.

Callus DL is slightly firmer than callus KL, and fewer cells ruptured in suspensions made with DL callus on the fast drum. On the slow drum, however, KL callus grew better than DL callus, and more E-cells and embryoids formed in the KL callus on the slow drum. Cells remained suspended on the fast drum, but tended to settle to the bottom of the tube on the slow drum and move as a group as the drum revolved. Thus, the cells that were less agitated on the slow drum may have had a better chance to differentiate or develop into embryoids. This does not explain, however, why the KL cells differentiated better than the DL cells on the slow drum, unless it was due to the carry-over of auxin from medium 17 by the DL callus. Residual auxin might have inhibited E-cell and embryoid initiation.

The currently popular theory of morphogenesis states that cells differentiate under the influence of cytokinin, but cell differentiation is inhibited by auxin. Thus, the auxin treatments should not have produced E-cells or embryoids.

One explanation is that, since E-cells and embryoids were already found in KL callus before it was placed in liquid, the cells may have already been triggered to differentiate and in liquid the auxin simply stimulated the development of the triggered cells. On the other hand, callus DL was grown on medium 17 containing auxin and no cytokinin, and did not have preexisting differentiated cells, and therefore the cells may not have been triggered before they were placed in liquid suspension. Callus DL cells would therefore have had to be triggered in suspension by cytokinin. However, cytokinin was not present and the auxin treatments inhibited the triggering of differentiation in the suspension cultures.

The next step would be to wash the callus tissue of all hormones, then add either cytokinin or auxin. This was done later, and is reported in a following section on washing.

REPLACEMENT OF AMMONIUM NITROGEN IN DOUGLAS-FIR SUSPENSIONS

Introduction

When aspen was propagated in our laboratory by tissue culture (22), the aspen callus had never been grown on a medium containing ammonium. Without ammonium ions the callus was sensitive to light and would turn red and die with a few days exposure. When we added ammonium nitrogen to the medium we could grow callus in the light and the callus turned green and survived. However, no shoots developed.

During numerous studies with suspension cultures of Douglas-fir and loblolly pine, there has seemed to be something present that inhibited the formation of true embryos and plants. In this study we investigated the possibility that ammonium ions inhibit cell differentiation, so we replaced not only ammonium nitrogen, but also all other forms of nitrogen including nitrates and amino acids.

In our first experiments we replaced ammonium nitrate with other forms of ammonium such as carbonate and phosphate, and in later experiments we replaced inorganic with organic forms of nitrogen.

Alternate Forms of Inorganic Nitrogen

In this study, all treatments were duplicated and two basic media were used for suspensions of DL3 callus growing on medium 17. The two basic media were liquid 17 made with 0.5 mg/liter 2,4-D and medium 26-10 with half-salts and no hormones. Medium 17 is usually made at pH 8, but all variations of 17 and 26-10 were adjusted here to pH 6. In earlier tests we found that with any starting pH of 5-8, the value usually stabilized at pH 4 after a day or so. We used medium 17 at both pH values, and all variations of 17 contained 2,4-D. Table V shows the alternate forms of inorganic nitrogen used in the two treatments, which were adjusted to correspond with the original amount of each form of nitrogen in the two media.

All tubes of suspension were placed on the fast drum (20 rpm) in continuous fluorescent light at 5000 lux and were examined at 1, 2, 5, and 9 days. After nine days, all suspensions in medium 17 and its variations were terminated and given to Johnson for biochemical analyses. The suspensions in medium 26-10 and its variations were continued and examined at 21, 29, 44, and 50 days.

Results

A few small embryoids were observed in suspensions of 17-8 and 17-J on the ninth day, but only rare E-cells were found in other variations of medium 17. Among the 26-10 media, there were only scattered E-cells found in the control suspension after nine days, but there were 60% E-cells and a few embryoids in

medium 26-1 made without ammonium and containing only nitrate nitrogen. There were only rare E-cells and no embryoids found in other variations, and this same pattern persisted until 44 days. No E-cells or embryoids were found in any suspension after 50 days.

TABLE V

ALTERNATE FORMS OF INORGANIC NITROGEN IN MEDIUM 17 AND 26-10

Component	17-8	17-J	17-1	17-2	17-3	17-4	26-10	26-1	26-2	26-3	26-4
NH_4NO_3	x	x					x				
KNO_3	x	x	x	x			x	x	x		
KH_2PO_4	x	x	x	x		x	x	x	x		x
KCl					x	x				x	x
$(NH_4)_2HPO_4$					x					x	
$(NH_4)_2CO_3$						x					

Note: Medium 17-8 was made at pH 8 and 17-J and all other media were made at pH 6. Ammonium carbonate was dissolved in water and filter-sterilized into autoclaved medium.

These combinations gave the following parameters:

	Ammonium	Nitrate	Potassium	Phosphorus
1.	0	-	x	x
2.	0	x	+	x
3.	x	-	x	+
4.	x	-	x	x

Note: 0 = none, - = less, x = same, + = more.

In this study, ammonium ions appeared to inhibit the formation of both E-cells and small embryoids among callus cells of DL3 dispersed in liquid medium 26-10. The removal of ammonium ions from the medium resulted in a high frequency of E-cells after nine days, but only a few embryoids were produced. This is the best performance for callus DL3 growing on medium 17 before transfer to liquid, but still does not equal the best performance of KL callus placed in liquid medium 26-10 containing ammonium ions.

Replacement of Inorganic with Organic Forms of Nitrogen

At this point in the research we suspected that not only ammonium, but also nitrate nitrogen might inhibit cell differentiation. In order to investigate this possibility we made up ten varieties of medium 26-10 without any inorganic nitrogen, but containing one or more of five alternate organic sources of nitrogen, either alone or in combination (Table VI).

Results

After two days, slight differences started to show up in cells among treatments. However, by 1, 2 and 3 weeks the differences were less and none of the treatments were any better than control medium 26-10. The autoclaved-urea medium 11 was contaminated with bacteria on the second day, indicating poor filter-sterilization. All other suspensions were discarded after two weeks with no significant differences and a smaller test of filtered vs. autoclaved urea was run.

Filtered vs. Autoclaved Urea as the Nitrogen Source

Medium U-3 was made again (Table VI) with 1000 mg/liter urea, but half was autoclaved and half was filter-sterilized into autoclaved medium. Control media were 17-8 and 26-10. Each medium was tested with three different callus

sources including DL3 and KL17 on medium 17, as well as KL13-MO on medium 3. Tubes were placed in continuous light on the fast drum.

TABLE VI

MEDIUM 26-10 VARIATIONS WITH ORGANIC FORMS OF NITROGEN[a]

Medium	Casein Hydrolyzate	Urea	Adenosine	Guanine	Adenine
1	1000				
2	1000		10		
3		1000			
4		1000	10		
5			10		
6			100		
7				10	
8				100	
9					10
10					100
11		1000[b]			

[a] Medium 26-10 was made without ammonium or potassium nitrate, and 2901 mg/liter KCl were added. All figures are in mg/liter. Media 1-10 were autoclaved.

[b] Urea was filter-sterilized into autoclaved medium.

Results

After 4 and 7 days, small embryoids and E-cells were observed in medium made with filtered urea but not with autoclaved urea. This was similar to the results in medium 26-10, but no E-cells or embryoids were found in medium 17.

These results indicate that urea may be a good alternate source for inorganic nitrogen, but possibly at lower levels than at 1000 mg/liter.

We also tested filtered vs. autoclaved arginine, but no consistent differences were found at 3, 6, 10, 13, 20 and 34 days. Neither were E-cells or embryoids found in arginine media made with 1000 mg/liter.

Filtered Urea and Arginine at Different Levels

The liquid media containing 1000 mg/liter filtered urea or arginine were diluted with control medium without any nitrogen source, to levels of 1000, 500, and 250 mg/liter of urea or arginine. Callus DL3 was placed in duplicate treatment tubes as well as in control medium 26-10 made with inorganic nitrates and ammonium. Tubes were placed on the fast drum in the high light source.

Results

After 10 days, a few pre-E cells were found in 500 and 250 urea suspensions and only rarely in the 250 arginine suspensions. After 14 days, 1000 urea was greener than 500 urea, but both were greener than the arginine tests. No E-cells were found except 1% in 250 arginine, along with browner cells. A few embryoids were found in the control 26-10 after 17 days, but none were found in any of the test suspensions. The 1000 urea remained greener than any arginine during the 31 days of the experiment, indicating that the lower level of urea may have been used up during the first 10-14 days of the test. Perhaps the 500 level would be better than 1000, but at more frequent intervals. The value of urea may be in keeping the cells alive longer than in control medium (which started to die in 31 days), allowing some other factor to help stimulate embryoid development.

Standard Media With and Without Urea Replacement

During April and May, several experiments were run utilizing media 17, 19 and 26-10 made with either normal nitrate and ammonium or with 500 mg/liter filtered urea. The main difference between medium 19 and 26 is that 19 contains full salts and 26 has half-salts (Tables II and VIII). Thus, 5800 and 2900 mg/liter KCl, respectively, were added to the two media along with the filtered urea, to replace the original amount of potassium present in the normal media. Medium 17 is medium 19 with 0.5 mg/liter 2,4-D. We thus tested medium 17 and 17U, 19 and 19U, and 26 and 26U. All media 17 and 19 were made at pH 8 and medium 26 was made at pH 6. Effects of pH will be discussed in a later section.

Results

Not all combinations were tested together in one experiment, and 19 has not yet been tested against 19U in cell suspensions. However, their characteristics are reported later as the effects of pH drops during their first 72 hours in suspension with cells.

From the first tests, the results showed that growth and differentiation were stimulated by medium 19U, after 2-3 weeks in suspensions made with DL3 callus and when the medium was left unchanged for two weeks. On the other hand, when the medium was changed after six days, cells differentiated better in medium 26-10. Using KL callus grown on medium 17, medium 26U stimulated cell differentiation in suspensions better than 19U when the medium was left unchanged, but again 26-10 was best when the medium was changed after one week. Medium 17U did well when the medium was changed for callus DL3, but not as good as 26-10. When the medium was changed for callus KL-17, the number of E-cells and embryoids in 17U were almost as many as in 19U suspensions after 3-4 weeks. But generally, medium 19U was better than 17U and 26U.

When callus KL-MO was used, grown on medium 3 with both auxin and cytokinin, 26U did better than the rest, but this callus must be placed on a slower drum to keep from rupturing and dying in suspension. Either low or high light will produce differentiated cells from KL-MO callus, but the slow speed is essential. In later tests, about equal performance was found among 26-10, 26U, and 19U at pH 6 (19-6U). However, 19U at pH 8 (19-8U) was better for callus KL or DL3 grown on medium 17 normally made at pH 8. These relationships will be discussed in a later section. We will also report that KL-MO callus will remain viable at drum speeds up to 10 rpm.

On the basis of these many tests, we have adopted medium 19-8U as a standard medium for inducing E-cells in suspensions using callus DL3 or KL-17, or for washing these callus sources or for enhancing the growth of E-cells into embryoids in suspensions.

EFFECTS OF MISCELLANEOUS INTERNAL AND EXTERNAL FACTORS

Introduction

In addition to replacing inorganic nitrogen with an organic nitrogen form such as urea, we have also studied the effects of various other factors relating directly to the cells in suspension, the nutrient medium, or the physical environment. A list of factors we have studied separately or in combination includes:

1. Initial pH and changes in culture
2. Washing out of possible inhibitors
3. Sieving for specific sizes of cell clusters
4. Flask vs. tube vessels
5. Drum vs. shaker vs. speed of rotation
6. Duration of culture before replenishing nutrients
7. Quality and quantity of light and temperature.

Effects of pH

In the previous section on alternate nitrogen forms in the nutrient medium, Table V shows that regular medium 17 is made at pH 8. However, medium 17 was also provided at pH 6, as were all its variations, to conform to the pH of medium 26-10. A second experiment was run to test the effects of pH on cell differentiation of callus KL17 in variations of medium 17, made at both pH 6 and 8. Suspensions were made in the same way, with one drop of callus per 10 ml medium per duplicate tube per treatment, all on a fast drum at 20 rpm in continuous light.

When examined after one day, a few embryoids were found in control medium 17 at pH 6 but not at 8. After 12 days, both of these suspensions had 5-10% E-cells but no embryoids. However, among the variations, 20-30% dividing E-cells were observed in variations 1-3 at pH 8 but not at 6. These results may be partially explained by the tendency of callus KL17 to produce some E-cells in callus cultures, but then E-cells should have been found in all suspensions. They may also confirm the fact that callus KL17 prefers pH 8 to 6 in both callus and suspension cultures. One confounding factor was the presence of bacteria in some of the pH 8 suspensions with E-cells, but the absence of bacteria in any pH 6 suspensions. However, this may only reflect the preference of bacteria for one pH over another, which may coincide with the preference of the callus cells.

In a recent major experiment, we looked at the immediate pH drop of several suspension cultures made with organic or inorganic nitrogen. Last year we looked at the immediate pH drop only with inorganic nitrogen, and found that pH drops rather predictably during the first 24 hours in liquid cultures from pH 6 to 4 in medium 26-10. We believe this drop is associated with an exchange of potassium ions from the nutrient with hydrogen ions from the cells, but others

think it is associated with sugar breakdown. We have not yet tested these hypotheses, nor have we run long-ranged pH tests with organic nitrogen vs. cell differentiation.

In this experiment we used medium 17 and 19, both with and without inorganic nitrogen replacement with urea. Medium 17 has 0.5 mg/liter 2,4-D and medium 19 does not. Otherwise they are identical. Two callus sources were used: DL3 and KL17. Four drops of callus were added to each duplicate tube per treatment, and pH values were measured.

The graphs of the pH readings are not shown here, but were recorded hourly for the first eight hours and hourly or bihourly the second day for 24-32 hours. Graphs of the two callus sources can be nearly superimposed on each other for the same medium. However, there are differences between media. The four media were made to pH 8 before autoclaving, but were all about pH 7 after autoclaving and before callus was added. For media 17U and 19U, 500 mg/liter were filter-sterilized directly into the medium after autoclaving; however, this did not cause a pH change.

Within the first hour, 17 and 19 both dropped 0.6 pH units and 17U about 0.4. Medium 19U only dropped 0.1. Thereafter, 17 and 19 dropped uniformly during the day to pH 4.7-4.8 after eight hours. Medium 17U turned up from 5.7 to 6.3 during the seventh hour, and 19U only dropped to 6.1 during the whole day. During the second day all curves stayed around pH 4, except for 17U which fluctuated between 6.9 and 6.1. At 48 and 56 hours, all suspensions were pH 3.5 and all were discarded. Our plans include rerunning these media for 2-3 weeks, with only weekly readings to compare with cell differentiation. However, we have many experiments already showing cell differentiation in these media but without the pH readings at weekly intervals.

From several other tests with regular or urea media at pH 6 or 8, we get fairly reproducible results that indicate that for cell differentiation medium 19U at pH 8 (19-8U) is better than 19-6U for callus KL17, which is also better than 17-8 or 17-8U. However, callus DL3 grows longer and greener as uniform undifferentiated cells in medium 17-8 better than in any other medium except possibly 26-10. For callus KL13-M0, however, previously grown on medium 3, embryoids will grow about equally well in liquid medium 3 without hormones, 26-10, 26U, and 19U.

Washing Out of Possible Inhibitors

In tests last year, some additives dropped onto callus stimulated growth, but so did the water control without additive. This was interpreted as a possible washing out of inhibitors such as phenolics or hormones. Recently, we started a new series of studies to determine the degree of washing necessary to cause the optimum growth or differentiation response. We have washed and not washed callus placed in liquid suspension, and have also combined this with testing other factors such as how much callus to add to the liquid, liquid media with and without urea and with and without hormones, light, drum speed, and replenishing all nutrients or just sucrose after different intervals. We have also washed up to 22 different callus lines and grown them in a hormone medium for three days, followed by washing and resuspension in medium without hormones.

From the many tests we have run, a general rule seems to be emerging that at least two washings by decanting are necessary to remove hormones from callus cells. However, the second washing also may be removing necessary nutrients from the cells by leaching. In some cases, where the decanting drains off all liquid, one washing seems better than two. A new series of tests is planned to determine if centrifugation at low speeds will remove all hormones, but fewer nutrients, with one wash.

Sieving for Cell Clusters

Most workers, producing embryoids from carrot suspensions, filter out clumps of cells smaller than 100-120 μm but larger than 50-60 μm. This seems to remove small cell clusters which do not form embryoids, as well as removing large clumps that may change critical hormone ratios in the suspension. We have used sieves to remove all large clumps, but only rarely have recovered specific fractions. One method is to simply shake soft callus in liquid medium, allow it to settle for different intervals, and decant the suspension. This will allow a rough division of clump sizes, but is difficult to reproduce. We have also used standard Tyler sieves, but are fearful of metallic poisoning from the screens. We have recently received nylon material woven to precise pore sizes down to 50-100 μm. This is used extensively in Japan, and we have just learned of an outlet in this country. We plan to make more use of the nylon material in the future to study cell-clump fractions in suspensions.

Other Factors

Most of the other factors have been tested in combination with nutrient, pH, and washing experiments, and will be reported here as general findings. Test tubes are preferred over flasks, mainly because they are easier to work with in large numbers and seem to give more reproducible results. Tubes are rotated on drums at 1, 10, or 20 rpm, and usually are placed in continuous light of 4000 lux, although some low-light tests have been made with continuous and interrupted light.

As a general rule, the callus cultures grown on medium 17 may be placed in liquid medium and rotated at 1 or 20 rpm and grow well. However, callus KL13-MO cells will generally rupture at 20 rpm. A variable-speed drum was purchased and run first at 20 rpm, then at 10 rpm, where both sources of callus cells grow well without harm. High light can be tolerated by the DL3 or KL17 callus cells in liquid,

but KL13-MO cells seem to prefer lower light of a few hundred lux. For the KL13-MO, cell differentiation is more apparent than in the other tissues, and differentiating cells may have a lower light tolerance. Full shade, however, seemed too dark. Further studies need to be run on the quality and quantity of light for growth and for embryoid initiation.

For continuous cultures of uniformly undifferentiated cells, callus DL3 in medium 26-10 seems best, with weekly changes of all the old medium. However, for differentiation of cells, if most cells are not E-cells, within the first week the liquid can be decanted to one-half its volume and an equal amount of fresh medium added. If all the medium is changed, dedifferentiation occurs and most cells become undifferentiated large-vacuole cells. The general cell cycle seems to be about nine days, but we do not know the frequency of synchronization of cell divisions. There does seem to be material in the liquid, either from other cells that have broken down or from exudates from living cells, which material seems to be feeding cells and causing differentiation. We have also started studies where only sucrose is added to the medium at intervals. However, results from these studies show no definite pattern as yet.

We have again tried cultures in the dark (wrapped with foil) with those in the light, and have combined these tests with short exposures to far-red light. We have seen some response, but have not been able to evaluate fully the impact of far-red on cell differentiation. After exposure to far-red, the cells are grown for several weeks in foil. We have not been able to do as much work as we wish with tungsten light or different temperatures with suspension cultures.

FEEDBACK RESEARCH

Introduction

In this category are included experiments at the suspension culture level designed to test the effect of chemical additives or physical manipulations that are derived from observations in other parts of the overall program. These experiments are generally conducted in the Tissue Culture Laboratory in 10 ml suspension cell cultures on rotating fast drums in the light. Evaluation is principally by frequent light microscopic examination, but occasionally there are also pH tests and chemical analyses conducted on spent media.

During the past two years many chemicals have been added to callus tissue, principally as drops onto the callus or as injections into the central part of callus pieces. Two chemicals showing promise during these preliminary tests were thiourea and hydrogen peroxide. In the tests that followed, various levels of these substances were added to liquid medium 17, 26 and 19U to study their ability to keep the Douglas-fir suspension cells green for long periods of time and to initiate E-cells.

Thiourea Additive

To 9 ml of medium 26-10 per tube, 1 ml aliquot of stock solution was added by filter sterilization to each tube to give treatment replicates of 0, 0.01, 0.001, and 0.0001M thiourea. A separate set of treatments was prepared for medium 17. Cytological examinations were made 1, 3, 6, 8, 10, 14, 16, 20, 23, 28, 51, and 65 days after the experiment started. One tube of each duplicate set was left with its original medium, but the second tube was decanted and fresh medium 17 or 26 (without thiourea) was added on the tenth day. Five ml of either medium 17 or 26 was added on day 20, and the medium was replaced on day 51.

The results showed that on the eighth day, small embryoids were found in suspensions made with medium 17 with thiourea added but not in medium 26 with thiourea. The optimum level of thiourea was 0.001M.

Thiourea and Hydrogen Peroxide

In a similar experiment, different levels of thiourea and hydrogen peroxide were added to medium 17 or 26 either singly or in combination. At 0.01% hydrogen peroxide, 50% dividing E-cells and scattered small embryoids were observed after six days in medium 26. However, only about 30% E-cells were found in the same treatment in medium 17. Thiourea did not do well alone in either medium, and a small response was noted in medium 26 with both additives. A few embryoids persisted until nine days in some suspensions already discussed, but for 0.01% hydrogen peroxide in medium 26, about 75% of the light-green cells were in the form of small embryoids. This was one of the few times that hydrogen peroxide gave such good results.

Continuing this experiment, all tubes were switched to the slow drum after nine days. With 0.001M thiourea in medium 26, about 60% E-cells were produced between day 34 and 43, but the same suspension only had 1-10% between 12 and 20 days. On the other hand 5×10^{-4}M thiourea and 0.015% hdrogen peroxide in medium 17 had 20% E-cells from day 12-15, 60% on day 20, and 1% on day 34. The best hydrogen peroxide alone was 0.001% in medium 26 which had 30% E-cells on day 20 plus a few embryoids, and 20% on day 34. Fresh basic medium was added to all tubes on day 43 and 82. After 82 days, most suspension cultures had died, but a few hardy cultures were still healthy. A few cultures in medium 17 were uniformly light-green and consisted of spherical cells that were not differentiated. These were combined and are still subcultured monthly to fresh medium 17 as a uniform stock culture. Some cultures were returned to agar medium to start new callus cultures from suspensions.

In the most recent study we wish to report, either thiourea at 0.001, 0.005, or 0.01M or hydrogen peroxide at 0.005, 0.010, or 0.015% were added to medium 26, 26U, 17, or 19U. Every week the suspensions were observed, fresh urea was filter sterilized into control and treatment suspensions made with 19U and 26U, and the original amount of hydrogen peroxide was replenished to those tubes originally made with this additive. The treatments were observed at day 6, 9, 13, 20, 27, and 34, and the best results are shown in Table VII. Using callus DL3, as usual, the best green color after 13 days was in medium 19U alone, followed by 26U plus 0.001M thiourea, and the best E-cells and embryoids were in 26U plus 0.001M thiourea followed by 26-10 plus 0.01M thiourea (Fig. 31-36). In this case, the half-salts of 26U did better than the full salts of 19U for differentiation, but not for green cells. After 27 days, there were more embryoids in 19U alone than in 19U plus thiourea. However, 19U plus 0.001M thiourea was the only treatment to have any embryoids after 27 days.

The results of this particular series of experiments seem to indicate that neither hydrogen peroxide nor thiourea stimulate the formation of E-cells or embryoids any better than 26-10 or 19U alone at longer times. However, both additives did produce E-cells at shorter times than did plain media and both do seem to stimulate cell differentiation better in the presence of urea than in the presence of nitrate and ammonium nitrogen. With further refinements of our suspension media we may see these additives contribute to our knowledge of the metabolic pathways involved in cell differentiation in both liquid and callus cultures.

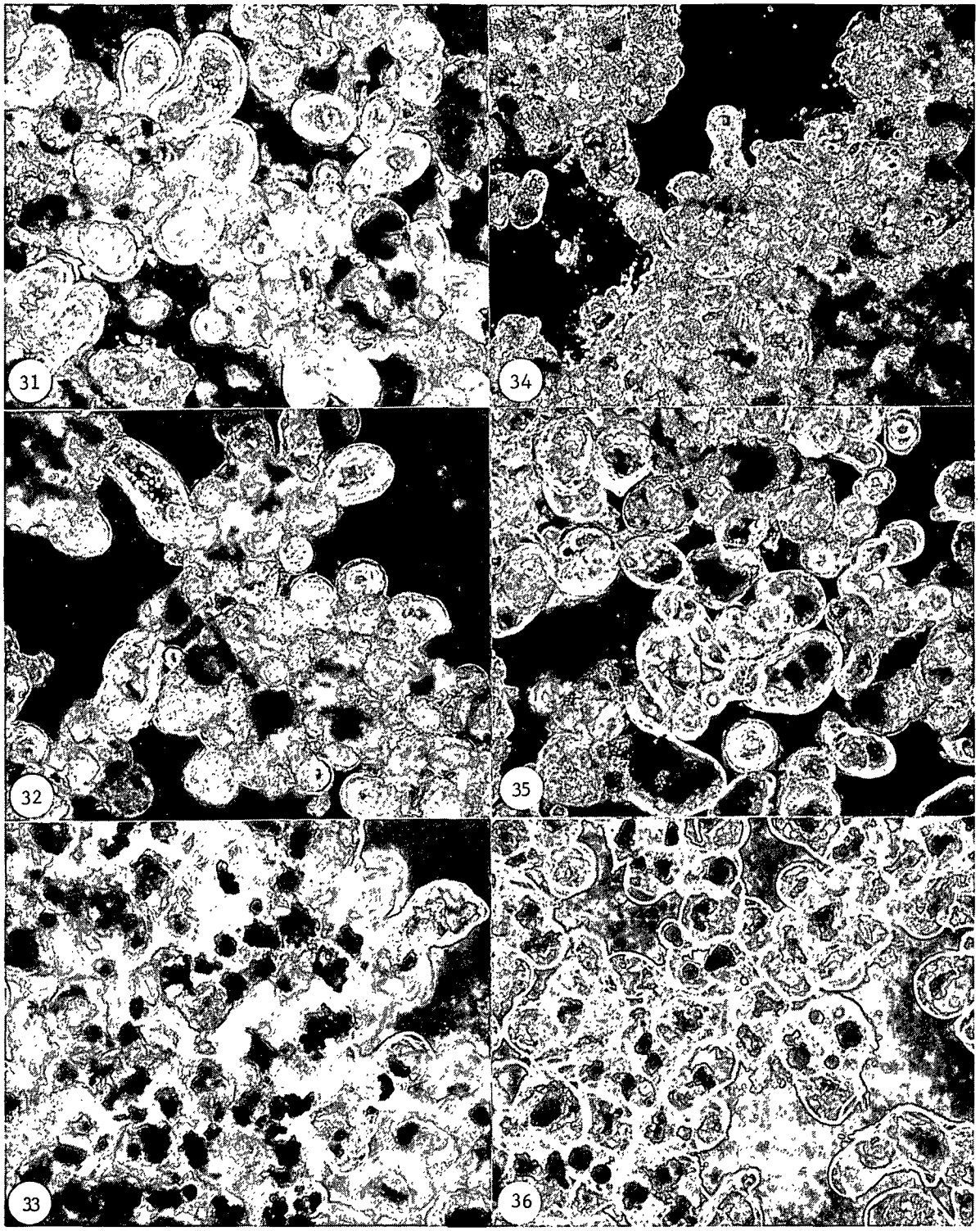
Thiourea was added to suspension cultures of Douglas-fir originally as a catalase inhibitor (43) because our enzyme investigations suggested that catalase activity might be excessively high in stem callus tissue. Results may still be interpreted on that basis, but it must be kept in mind that other explanations may be possible in place of or in addition to catalase inhibition (e.g., see

TABLE VII

BEST TREATMENTS FOR GREEN OR DIFFERENTIATED CELLS IN SUSPENSION CULTURE OF DOUGLAS-FIR CALLUS IN FOUR LIQUID MEDIA[a]

Medium	Additive	Level	Days After Start					
			6	9	13	20	27	34
17-8	None	0	LG	LG	LG	VLG	T	G
	Thiourea	0.001M	LG		LG			
		0.005M		LG		VLG	T	--
	H_2O_2	0.005%	LG	T	LG	T	B	--
19U	None	0	G	G	G	G,E	E	--
	Thiourea	0.001M	G	T	T,E	G,B	B,E	--
	H_2O_2	0.001%	LG	G	G,T	LG,E	B	--
26-10	None	0	G	G	LG	G,E	LG	G,B
	Thiourea	0.001M	G					
		0.005M			G	LG,E	LG,B	--
		0.010M		VLG,E	LG,E	LG,E	--	--
	H_2O_2	0.005%		T,E				
		0.001%	G,E		LG,E	B	--	--
26U	None	0	G	G	G	BG	G	G,B
	Thiourea	0.001M		G	G,E	LG,E	B,E	--
		0.005M	LG					
	H_2O_2	0.005%	LG,E	LG	LG	LG,T	G	--

[a]Callus DL3. LG = light green, G = green, BG = bright green, T = tan, B = brown, VLG = very light green, E = E-cells, E = embryoids, -- = dead or discarded.



Figures 31-36. Callus DL3 in Suspension Cultures After 27 Days in the Light on a Fast Drum at 20 rpm. Figure 31. Medium 19U. Figure 32. Medium 19U + 0.001M Thiourea. Figure 33. Medium 19U + 0.005% H_2O_2. Figure 34. Medium 26U. Figure 35. Medium 26U + 0.001M Thiourea. Figure 36. Medium 26U + 0.005% H_2O_2. All 140X

STUDENT RESEARCH following), particularly since rather high thiourea concentrations were required to demonstrate inhibition in on-gel staining following anionic electrophoresis and then all isoperoxidases were inhibited (Fig. 37). Thiourea initially seemed to promote the formation of E-cells and embryoids in a reproducible manner at a concentration in the vicinity of one millimolar; nevertheless, from later experiments it appears that the thiourea concentration may be rather critical relative to the history and condition of the inoculum and also relative to suspension medium composition. Hydrogen peroxide has been used with similar results. Here the optimum concentration seems to be 0.01-0.001%. Rationale for its use is similar to that for thiourea, but hydrogen peroxide attacks the same problem from a different direction, i.e., an attempt to keep ahead of catalase destruction and provide oxidizing agent (not necessarily hydrogen peroxide itself) for other oxidases like IAA oxidase. Again it must be recognized that hydrogen peroxide alone is not a very specific oxidizing agent and that catalase will immediately reduce exogenous peroxide to a low level. Whether through immediate oxidation or through continued oxidation by catalase residual, the peroxide may be acting on something other than or in addition to IAA. A very important aspect of the action of both thiourea and hydrogen peroxide additives could be a lack of peroxide production in stem callus. Tests for peroxides in both organized and unorganized tissue were conducted, but so far interference in the test prevents any conclusions.

The use of arginine and urea as nitrogen sources in feedback experiments with both callus and suspension cultures was mentioned earlier in the report. What may not be obvious is that urea could be formed in the same enzymatic reaction in which ornithine is produced from arginine. Both urea and arginine will serve as nitrogen sources. Whether they will be better than ammonium and nitrate combinations in attempts to induce and continue embryogenesis remains to be seen.

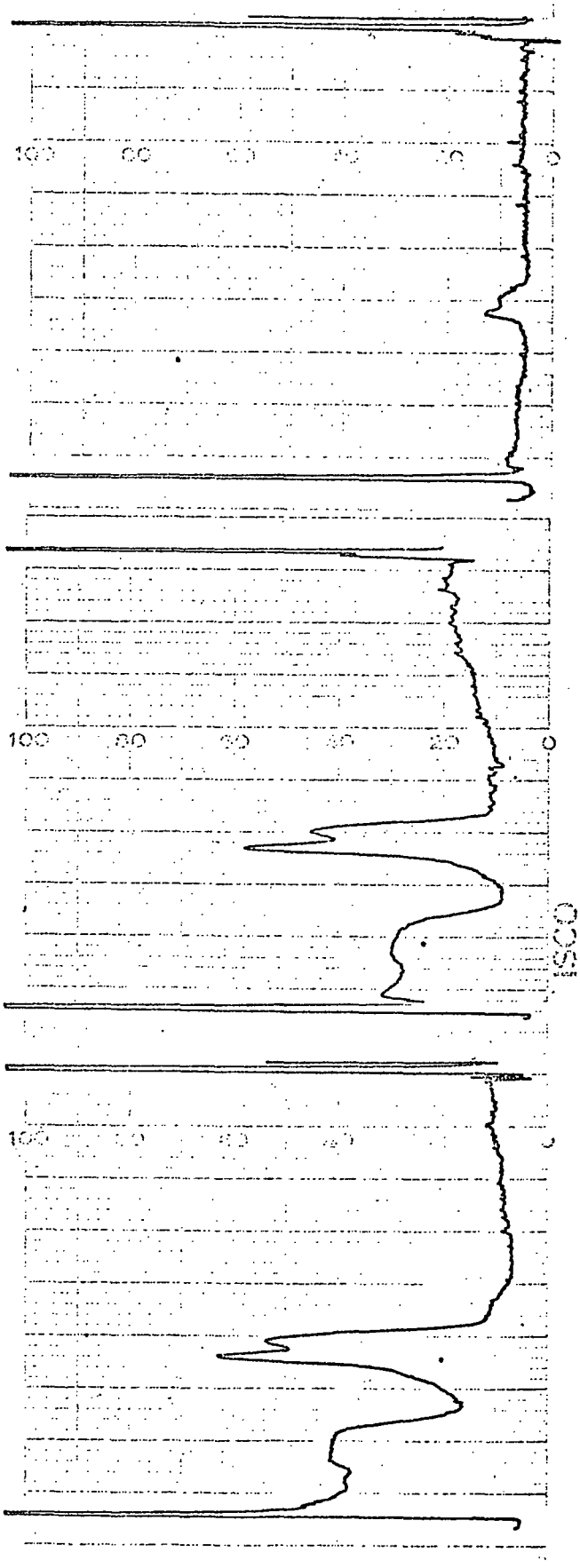


Figure 37. Effect of Thiourea Upon Peroxidase-Catalase Activity after Anionic Electrophoresis:
(a) No Thiourea, (b) 1 mM Thiourea, (c) 10 mM Thiourea

EMBRYOIDS FROM NEW DOUGLAS-FIR AND LOBLOLLY PINE CALLUS

Douglas-fir

In Progress Report Three, January 30, 1976, are shown several large embryoids of Douglas-fir, some composed of over one hundred cells with small vacuoles (E-cells). During this past six months, we have deemphasized the production of large embryoids from old stem callus, and have done more work with feedback studies. However, some callus from new stem callus cultures has been used to produce small embryoids in suspension cultures. The new callus has been used in studies where the most callus was from old cultures of mainly Douglas-fir, so only a few experiments were run using exclusively new callus. In one such study, new Douglas-fir callus was used that had been subcultured eleven times from two-year-old seedlings onto medium 10 at monthly intervals. The callus was grown in an incubator with continuous light at 5000 lux at 25°C, then for the last two passages for 16 hours at 5000 lux at 24°C, alternating with 8 hours dark at 18°C. Callus was grown in duplicate tubes of 10 ml each, in four media and on four drums at different speeds and light.

Medium 3 is medium 10 (Table VIII) with 1 mg/liter NOAA instead of 5. Callus was grown in medium 3, medium 26-10, and two others testing nitrogen and hormone relationships. The drums were operated at 1, 10, and 20 rpm in 5000 lux, and 1 rpm at 2000 lux fluorescence. Callus cells formed radiating embryoids from cell clumps in six days (Fig. 38-39). These were in medium 3 at 10 rpm in full shade under 5000 lux and in medium 26-10 which persisted for 14 days.

Callus from new Douglas-fir cultures is now becoming soft green and is growing in sufficient quantities that we can now use some occasionally for suspension or callus tests aimed at inducing embryoids or shoots.

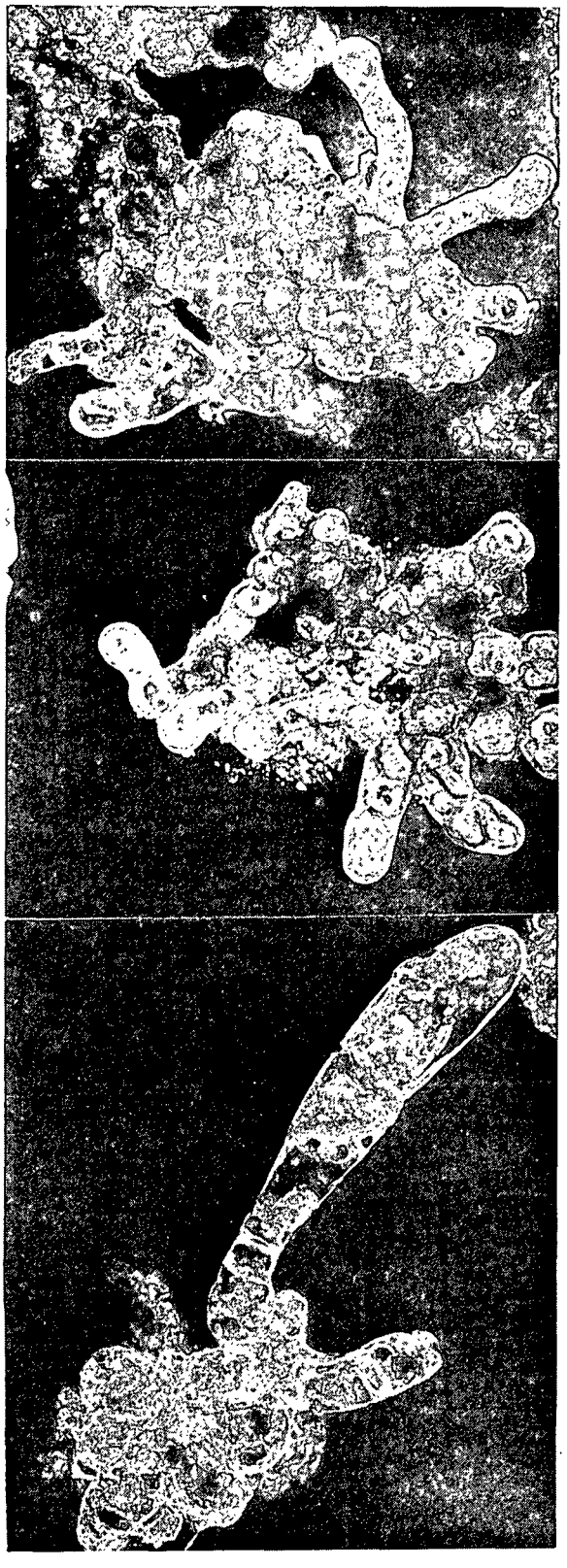


Figure 38. Radiating Embryoids after Six Days in Medium 3 at 10 rpm in Shaded 5000 Lux. Callus was Initiated from Stems of Two-Year-Old Seedlings of Douglas-fir and Grown for Eleven Subcultures on Medium 10. 140X

Figure 39. Shorter Embryoids after Six Days in Medium 26-10 at 20 rpm in 5000 Lux. Callus from the Same Douglas-fir Source as in Fig. 1. 140X

Figure 40. A Medium-Sized Embryoid after 54 Days in Suspension in a Variation of Medium LV-10. Callus was Initiated from Stems of a One-Year-Old Seedling of Loblolly Pine and Grown for Nine Subcultures on Medium 10 and for Two on Medium LV-10. 140X

Loblolly Pine

We have occasionally observed embryoids in suspensions made with loblolly pine stem callus. However, with the problems we have been having with the callus medium (see Section 2, Optimizing Growth Medium for Loblolly Pine Callus), not too much new callus was available for studies of shoot induction or for cell suspensions. Also, the callus was in only fair to poor condition and was tan-green instead of bright-green in color. Recently, Mrs. Verhagen has been working on medium LV-10 and prepared some of one of her variations in liquid form. In this was placed new callus that had been through nine monthly passages on medium 10, then two passages on LV-10. It had also been grown in fluorescent-tungsten light and was light green and fairly soft. One tube each was placed on each of the four drums.

Occasional E-cells were found after 19 days in full shade under 5000 lux at 10 rpm, but cells in all except one culture turned brown after 30-40 days and were discarded. Cells in the slow drum in low light had many light-green cells and occasional embryoids composed of cells with large vacuoles (Fig. 40). The cell cluster at the base of the embryoid in Fig. 40 is characteristic of most large embryoids of either Douglas-fir or loblolly pine, and is composed of small cells including some with small vacuoles (E-cells). The embryoid shown in Fig. 40 is typical of those presently being found in suspension, having light-green color throughout the cell cytoplasm and appearing to be in good health. Occasional small E-cells also occur, but all large embryoids were of the large-vacuole cell types.

Future Plans

We are now growing a considerable amount of newly-initiated stem callus from Douglas-fir and loblolly pine. Although we believe that needle callus had a better promise for shoot propagation from callus, we nevertheless believe that

continued work with some new stem callus will contribute to our total understanding of the metabolism involved in shoot initiation from callus derived from several tree sources.

ORGANIZATIONAL PROCESSES: AN OVERVIEW

After more than a year of essentially analytical and probing research into the problem of organization of cultured conifer tissue, it became apparent that it would be necessary to stop looking for leads (at least temporarily) and focus more intently on some aspect of our then-current findings. Among our observations of on-gel enzyme electrophoretic distribution patterns, those of peroxidase were among the most interesting. It appeared that they might well be related to organizational state. Furthermore, the literature on isoperoxidases in developmental processes was continuing to expand rapidly. As more data accumulated, a working hypothesis began to take shape in which isoperoxidases and related enzymes would play a central role. It was natural to consider other enzymes that might be related to the peroxidases by a common product/substrate or vice-versa, and that accounts for most of the enzymes currently under investigation. Catalase was one of the first of these related enzymes to be examined bcause it is reported to have the capacity to act as a peroxidase as well as to destroy the hydrogen peroxide needed by peroxidases in order for them to function. As can be gathered from this report, catalase seems to be quite active in callus tissue relative to organized tissue. Another related enzyme which also needed investigation was IAA oxidase because of the possibility that it also functions as a peroxidase.

The foregoing enzymes either utilize or destroy hydrogen peroxide. Eventually it became of interest to ask questions like "Where is the hydrogen peroxide coming from if it's a problem?", "What are the consequences of its presence?", "Does the presence of large amounts of catalase imply that there is an abundance of hydrogen peroxide present that must be destroyed?", etc. We are still in the process of trying to answer such questions, but they have given rise to

further study of enzymes like phenol oxidases, polyamine oxidases, and superoxide dismutase. The observations that thiourea (a known catalase inhibitor) and hydrogen peroxide itself have given rise to E-cells and embryoids in feedback experiments on several occasions suggest that our current preoccupation with these enzymes may prove to be rewarding.

A major role is currently postulated for peroxidation and/or oxidation in the induction of embryogenesis or organogenesis with the possibility that nitrogen metabolism is a key factor in completing embryoid development. However, the latter may be mostly a case of optimization. Further amplification of these ideas may be expected in later reports and meetings.

STUDENT AND OTHER ONGOING RESEARCH

Student Research

As any major research program begins to develop, areas of related research become evident and it becomes appropriate to encourage student investigations of specific problems within these areas. This has been the situation with Project 3223. Currently, there are three Institute students (Bobalek, Smits and Mohler) working on studies that are related to Project 3223 research.

John Bobalek is working on the production of sterile protoplasts. This past year the enzyme solution was successfully filter-sterilized, cell walls were removed from Douglas-fir callus cells, cell walls were regrown, and the cells have divided and formed callus masses. Next fall, John plans to attempt fusion of protoplasts of different races, then will look for selection methods to recover fusion products. Since protoplast fusion will be the next major goal of this project, John's progress will be followed with great interest and is expected to give us a running start on this phase of the program.

Michael Smits has been working with the glyoxalase enzyme system from Douglas-fir stem callus. Both glyoxalases I and II have been found in the callus where glyoxalase I has about twice the activity as it does in organized tissue (whole seedlings) on a fresh weight basis. This is a two-enzyme system which requires glutathione; the latter has provided some interesting responses in perturbation research (cf. Report Three, p. 27, 29). Thiourea, which we have used ostensibly as a catalase inhibitor in suspension feedback experiments, may also inhibit this system, possibly through inductive effects on the substrate. Work with inhibitors of only glyoxalase II has also roused considerable interest and prompted some feedback research by Mr. Smits. While a check on reproducibility is still needed, his feedback run was promising in terms of an organizing response.

Another student, Michael Mohler, has just started research on methyleneoxindole, an IAA oxidation product. Methyleneoxindole has been claimed by some to be the active form of auxin while others have disputed the claim and suggest that it is really an inhibitor (45). Mr. Mohler was engaged in synthesizing methyleneoxindole during the spring term and so far his work has progressed well. It is hoped that some good product will be available for use in this project late this fall.

Ongoing Research

In addition to the studies reported in previous sections of the report, there are a number of investigations under way that are not sufficiently advanced to be reported in any great detail. One ongoing aspect of the program is to develop a technique for rooting shoots initiated from various types of callus. The following comments describe the procedures followed in the production and rooting of a cotyledon shoot. Embryos were excised from seeds of Douglas-fir on 3-17-75 and placed on agar medium 19 with 0.05 mg/liter BAP (benzylaminopurine). Multiple shoots appeared 3-4 weeks later and were excised after another four months and placed in an agar rooting medium with 10 mg/liter IBA (indolebutyric acid). After three months the shoots had not yet rooted and were transferred to medium without hormones, where they formed callus and grew additional shoots from their callus masses. On 4-15-76, about one year after shoots were first formed, one of the shoot clusters rooted. The root was 1 cm long one week later and covered with root hairs, and the tallest shoot was 7-8 cm. Nutrient agar medium appears to be a poor rooting medium, so we are currently testing other rooting media such as soil, peat, and sand-vermiculite, and dipping the basal end of juvenile cuttings into solutions of high levels of IBA (indolebutyric acid) to stimulate rooting. The effects of bottom heat and top light are also being studied.

Another ongoing study involves the work of optimizing the growth medium for loblolly pine callus. Mrs. Shirley Verhagen, using information in the literature, developed the LV medium that works best so far for loblolly pine stem callus. She will continue to put together a medium with all of the features she has found to work best during the past year, and will also test different light sources having fluorescent and tungsten light.

Perhaps our most important achievement during this past six months has been the recovery of one leafy shoot from subcultured needle callus of Douglas-fir. We believe that a practical method for propagating forest trees will first be propagation from needle callus, although mass propagation will later become more efficient from suspension cultures. We are now culturing needle callus from Douglas-fir and loblolly pine seed embryos and seedlings, and for Douglas-fir we also have needle callus from two-year-old and mature trees. We are treating the callus for shoot initiation, and some is also being used for suspensions.

Feedback research is another important part of our ongoing research program. After six months of intensive examination of additive studies, we now plan to put the feedback information to work in our suspension program, and will attempt to enlarge embryoids into true embryos and plants. We can now prolong the life of suspensions, and also believe we are close in being able to trigger enlargement. For this stage, however, we may have to look at multiple combinations of factors we have been studying separately or in combination of a few factors at once.

PLANS

Planned research during the coming six months is expected to cover a wide variety of topics. Comments regarding plans were given in preceding sections as part of the review of recent progress. Also, the section on "Ongoing Research" provides additional insight into several areas where additional information is required and work will be continued. To briefly summarize, plans for the coming six months are expected to include:

(1) Morphological and ultrastructural observations on developing embryoids and, hopefully, "student produced" fused protoplasts.

(2) Continued attempts to trigger shoot development from several sources (stem, needle and cotyledon) of loblolly pine and Douglas-fir callus.

(3) Additional studies on replacing ammonium nitrogen in cell suspensions with the objective of increasing the numbers of embryoids produced and overcoming the apparent inhibition of ammonium ions on embryoid development.

(4) Additional investigations into the optimum initial pH, methods of washing out inhibiting chemicals and techniques for sieving and recovering small cell clusters.

(5) Feedback research aimed at triggering embryoids in cell suspensions to enlarge and develop into true embryos and plants.

(6) Studies aimed at developing a reliable technique for rooting shoots initiated from various types of callus.

(7) Additional modifications of the present growth medium for pine in an effort to optimize growth of loblolly pine callus.

(8) Additional enzyme and tracer investigations having the objective of clarifying the interrelationships among ornithine, arginine, polyamines and polyamine oxidases.

(9) Additional enzyme and tracer investigations into the role of oxidases, peroxidases, catalase, and dismutases in organizational processes.

(10) Studies on the interaction of the foregoing enzymes with plant hormones.

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GLOSSARY

Amyloplast — A colorless plastid modified for starch storage.

Asexual reproduction — Reproduction without fertilization. New individuals may develop from vegetative parts such as tubers, bulbs or rooted stems, or from sexual parts such as unfertilized eggs or other cells in the ovule.

Auxins — A class of plant growth hormones of diverse makeup which cause cell enlargement, apical dominance and root initiation.

Callus culture — The dissociated component cells of a tissue growing on a nutrient medium.

Cell suspension — A liquid culture of small groups of cells agitated in some way to provide aeration and growing in a liquid nutrient medium.

Chloroplast — A membrane-enclosed subcellular organelle containing chlorophyll. Chloroplasts are the sites of photosynthesis. They contain DNA and polysomes and can replicate.

Clonal propagation — Propagation of a group of plants derived from a single individual (ortet) by asexual reproduction. All members (ramets) of a clone have the same genotype and consequently tend to be uniform.

Cytokinin — A class of plant growth hormones associated with cell division, assisting with the transmission of the genetic information from the genes to the proteins.

2D TLC — Two-dimensional thin layer chromatography.

Differentiated callus — Result of the process whereby cells in a callus mass divide to give rise to dissimilar, more specialized tissues.

Diploid — Having two sets of chromosomes in the nucleus. One-half of the chromosomes are contributed by one parent, one-half by the other parent. Many higher organisms are diploid except for their sex cells and associated tissue.

E-cells — Embryonic cells that have a relatively small central storage vacuole surrounded by a thick cytoplasm. Normal callus cells have a large vacuole and a small amount of cytoplasm.

EM — Electron microscope.

Embryo — A young plant developing from an egg cell, after ferilization, or without fertilization.

Embryoid — A cell group approximating an embryo, but having a more random cell arrangement.

Empirical method — Method based solely on experiment and observation.

Enzyme — A protein molecule that catalyzes a specific chemical reaction.

ER — Endoplasmic reticulum. A system of membranes (originating from the external membrane of the nuclear envelope) that permeates the cytoplasm and which may or may not be covered with ribosomes.

Eucaryotic cells — Cells with true nuclei bounded by nuclear envelopes and which undergo meiosis.

Fertilization — Sexual fusion of male and female nuclei.

Gene — One of the units of inherited material carried on a chromosome; arranged in a linear fashion and indivisible.

Gene pool — Reservoir of genetic variability available for use in genetic improvement of tree species.

Genetic gains — Average improvement in progeny over the mean of the parents.

Genetic variability — The variation existing in a given population (species, for example) with respect to particular genes or arrangement of genes.

Groundplasm — Homogeneous plasma (matrix) remaining after cell organelles and particles have been excluded.

Haploid — Having the reduced chromosome number, i.e., having one set of chromosomes in the nucleus. This is normal in sex cells, which have only half the number of sets occurring in diploid vegetative cells.

Hormone — Any growth substance which is generally transported to the site of action and can stimulate growth or cell enlargement (auxins), cell division (cytokinins), stem elongation (gibberellins), or can retard growth as in the abscission of leaves (ethylene).

Hybrid vigor — The increase in vigor, size and fertility of a hybrid as compared with its parents, resulting from the union of genetically different gametes and assumed to be due to special recombinations of dominant and recessive genes (heterosis).

Hybridization — The production of offspring of genetically different parents.

Hydrophobic — Water repelling.

Interspecific hybrid — The progeny from matings between species.

Intraspecific hybrid — The progeny from matings within species.

Lipids — Any of a group of biochemicals which are variably soluble in organic solvents like alcohol and barely soluble in water.

Meristem — A localized group of cells, actively dividing and undifferentiated but ultimately giving rise to permanent tissue such as shoots, roots, wood of bark.

Meristemoid — A localized group of cells in callus tissue, characterized by an accumulation of starch, RNA and protein, and giving rise to adventitious shoots or roots.

Mitochondria — Small bodies in spaces of the ground cytoplasm. They are spherical, long rods, or threads, and are the sites of many important enzymatic processes. The inner layer of the wall is infolded into fingerlike processes.

Nonrelated species — Species that are members of different genera.

Organelle — A complex xytoplasmic structure of characteristic morphology and function, such as a mitochondrion or plastid.

Parasexual hybridization — Hybridization resulting from asexual fusion of cells, either diploid or haploid.

Plasmalemma — The semipermeable, unit membrane surrounding and containing the cell cytoplasm. In plant cells, it is pressed up against the inner surface of the cell wall.

Polyploidy — Having three or more times the haploid number of chromosomes.

Procaryotic cells — Single-celled organisms and reproducing entities that lack a membrane-bound nucleus; they do not undergo meiosis; these include the viruses, bacteria, and blue-green algae.

Protoplast — Spherical cell protoplasm (cytoplasm + nucleus) bounded by a membrane but no cell wall.

Protoplast fusion — Union of two protoplasts into one cell.

Ribosomes — Macromolecules containing protein and RNA. They are seen as dense particles in electron micrographs. They are found in all types of cells in which protein is being synthesized.

SEM — Scanning electron microscope.

Somatic — Diploid body cells of an organism; those cells other than germ cells.

TEM — Transmission electron microscope.

Tissue culture — General term for callus and cell cultures of undifferentiated cells.

Ultrastructural — Sublight microscopic, intracellular structure.

Undifferentiated callus — A tissue composed of undifferentiated cells. The cells are thin-walled, capable of division and generally spherical and uniform in size. They have very little, if any, secondary wall thickening and no specialization such as characterizes fiber cells or conductive tissue. The chemical composition is generally that of xylem (wood) cambial cells found in trees.

Vacuole — A fluid-filled space in a cell. A single vacuole, taking up most of the volume of the cell, is present in many plant cells and contains a cell-sap which is isotonic with the protoplasm.

Vegetative cells — Nonreproductive cells such as haploid cells from female gametophytes of conifers or diploid somatic cells.

Vesicle — Small membrane-bound body in the cytoplasm.

Zygote — Fusion product of male and female sex cells or fusion product of protoplasts.

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APPENDIX

TABLE VIII

COMPONENTS OF NUTRIENT MEDIA IN MILLIGRAMS PER LITER

Component	IPA	3[a] & 10[a]	17[b]	26-10
NH_4NO_3	1,650	1,650	1,650	400
KNO_3	1,900	1,900	1,900	4,000
$MgSO_4$	370	370	370	185
KH_2PO_4	340	170	170	85
$CaCl_2$	440	440	440	220
$MnSO_4$	16.9	16.9	16.9	8.5
$ZnSO_4$	10.6	10.6	10.6	5.3
H_3BO_3	6.2	6.2	6.2	3.1
KI	0.83	0.83	0.83	0.41
Na_2MoO_4	0.25	0.25	0.25	0.125
$CuSO_4$	0.025	0.025	0.025	0.0125
$CoCl_2$	0.025	0.025	0.025	0.0125
Fe(EDTA)	5.6	5.6	5.6	5.6
Nicotinic acid	0.5	0.5	--	--
Thiamine	3.0	0.1	--	--
Pyridoxine	0.1	0.1	--	--
Folic acid	0.1	--	--	--
Riboflavin	0.1	--	--	--
Biotin	0.1	--	--	--
Adenine sulfate	100	--	--	--
Ascorbic acid	1	--	--	--
Inositol	100	100	--	--
Asparagine	100	100	--	--
Casein	--	--	--	--
Adenosine	--	--	--	--
Sucrose	30,000	30,000	30,000	35,000
Agar	8,000	8,000	8,000	--
BAP	--	0.1	--	--
IPA	0.01	--	--	--
NOAA	5	--[a]	--	--
2,4-D	--	--	0.5	--

[a] Medium 3 contains 1 mg/liter NOAA and Medium 10 contains 5 mg/liter NOAA. NOAA — naphthoxyacetic acid; 2,4-D — 2,4-dichlorophenoxyacetic acid; BAP — benzylaminopurine; IPA — isopentenylaminopurine.

[b] Medium E-1 contains Medium 3 salts, iron, 0.4 thiamine, 100 inositol, and 40,000 sucrose.

Medium 19 = 17 without 2,4-D.

TABLE IX

LIST OF ABBREVIATIONS

2,4-D	2,4-dichlorophenoxyacetic acid
ADP	adenosine diphosphate
ATP	adenosine triphosphate
arg	arginine
BAP	benzylaminopurine
BG	bud growth
cAMP	3',5'-cyclic adenosine monophosphate
CI	callus initiation
DF	Douglas-fir
IBA	indolebutyric acid
IPA	isopentenylaminopurine
LP	loblolly pine
MOPS	morpholinopropane sulfonic acid
MS	Murashige and Skoog
NAD^+	nicotinamide adenine dinucleotide (oxidized)
$NADP^+$	nicotinamide adenine dinucleotide phosphate (oxidized)
NBT	nitrobluetetrazolium
NOAA	naphthoxyacetic acid
OI	organ initiation
phe	phenylalanine
pro	proline
TLC	thin-layer chromatography
trp	tryptophan
trpAm	tryptamine
tyr	tyrosine